REGULATION OF AN OPERON CODING FOR THE COMPONENTS OF THE MOLYBDATE TRANSPORT SYSTEM (*modABCD*) BY MODE PROTEIN A MOLYBDATE-DEPENDENT REPRESSOR IN *ESCHERICHIA COLI*

By

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
A. vinelandii	*Azotobacter vinelandii*
AvModE	*Azotobacter vinelandii* ModE protein
bp	Base pair
C. pasteurianum	*Clostridium pasteurianum*
CpMopII	*Clostridium pasteurianum* MopII protein
CTP	Cytosine triphosphate
Da	Dalton
DNase I	Deoxyribonuclease I
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EcModE	*Escherichia coli* ModE protein
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FDH-H	Formate dehydrogenase linked to H_2 evolution
FeMoCo	Iron-molybdenum cofactor
FHL	Formate hydrogenlyase
GTP	Guanosine triphosphate

H. influenzae	*Haemophilus influenzae*
HiModE	*Haemophilus influenzae* ModE protein
HYD	Hydrogenase
IPTG	Isopropyl β D-thiogalactopyranoside
K_D	Dissociation constant
kDa	Kiledalton
Kinase	T4 polynucleotide kinase
Klenow	DNA polymerase I- Klenow fragment
LB	Luria Broth
LBG	Luria Broth + glucose
Mo	Molybdenum
MoCo	Molybdenum cofactor
ModE	ModE protein
MGD	Molybdopterin guanine dinucleotide
MPT	Molybdopterin
OD	Optical density
ONPG	Ortho-nitrophenyl-β-D-galactopyranoside
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
R. capsulatus	*Rhodobacter capsulatus*

RcMopA	*Rhodobacter capsulatus* MopA protein
SDS	Sodium dodecyl sulfate
TMAO	Trimethylamine N-oxide
Tris	Tris-(hydroxymethyl)-aminomethane
TTP	Thymine triphosphate
UV	Ultraviolet
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

LIST OF GENE SYMBOLS

All the genes listed below are from *Escherichia coli* unless otherwise indicated

Gene Symbol	Alternate gene symbols; phenotype affected
arcA	Aerobic respiratory control; putative DNA binding protein of Arc modulon
arcB	Aerobic respiratory control, histidine protein-kinase of Arc modulon
bla	β lactamase, ampicillin resistance
chlD	Chlorate resistance gene D [renamed as *mod*; molybdate transport]
fdhF	FDH-H formate dehydrogenase (linked to H evolution)
fhlA	Putative DNA binding protein necessary for transcriptional activation of the *fdhF* and *hyc* operons
fnr	'Global' regulator of anaerobic respiration; similar to cAMP receptor protein
galETK	Galactose operon
hyc	Hydrogenase isoenzyme-3
lsg	*H. influenzae* lipopolysaccharide synthesis gene
moa	Molybdopterin biosynthesis as chlorate resistance gene *A*
mob	Molybdopterin biosynthesis as chlorate resistance gene *B*
mod	Molybdate transport as chlorate resistance gene *D*
moe	Molybdopterin biosynthesis, as chlorate resistance gene *E*
mog	Molybdopterin biosynthesis, as chlorate resistance gene *G*

mop	Molybdopterin binding protein
narL	Putative DNA binding protein of respiratory nitrate reductase operon
narQ	Putative nitrate sensor for activation of the NarL protein
narX	Putative nitrate sensor for activation of the NarL protein
nit-1	mutation allele resulting in nitrate reductase deficiency in *Neurospora crassa*

Abstract of Dissertation Presented to the Graduate School
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The *modABC* genes encode a molybdate-specific transporter in *Escherichia coli* When the intracellular molybdate concentration is sufficient to support the activity of molybdoenzymes, expression of the *modABCD* operon is very low and increases under molybdate-deficient conditions. Isolation and characterization of a mutant strain (strain SE1811), which maintained the same level of expression of the *modABCD* operon in both the presence and absence of molybdate in the medium, resulted in the identification of a regulatory gene, designated *modE* The *modE* gene constitutes the first gene in a two gene *modEF* operon which diverges from the *modABCD* operon and codes for a 262-amino-acid protein (28 kDa) ModE, in the presence of molybdate represses the production of plasmid-encoded ModA and ModB proteins in an in vitro transcription-

translation system. DNA mobility shift experiments demonstrated that ModE is capable of binding as a dimer to DNA derived from the *modA*-operator/promoter region with an apparent K_d of 0.22 μM in the presence of 10 μM molybdate or with an apparent K_d of 1.3 μM in the presence of 10 μM tungstate. DNA-mobility shift experiments also revealed that a 50% shift of DNA is achieved at a concentration of 6 μM molybdate in the binding reaction. DNase I footprinting experiments identified three regions of protection by ModE in the *modA* operator/promoter region, 'GTTATATTG' at position -15 to -7, 'CCTACAT' at position -3 to +4, and 'GTTACAT' at position +8 to +14. In vivo ModE titration experiments confirmed that the indicated protected regions are required for efficient binding of ModE to DNA. A highly conserved amino acid sequence, TSARNQXXG (amino acids 125 to 133) was identified in ModE and homologs from *Azotobacter vinelandii*, *Haemophilus influenzae*, *Rhodobacter capsulatus*, and *Clostridium pasteurianum*. *E. coli* ModE mutant proteins with mutations in either the T or G repressed transcription of the *modABCD* operon in the absence of molybdate, suggesting that this stretch of amino acids is essential for binding molybdate by ModE protein. This study provides evidence that ModE serves as a repressor of the *modABCD* operon in response to intracellular molybdate levels.

INTRODUCTION

Molybdenum is required for the activity of several enzymes found in animals, plants, and bacteria such as sulfite oxidase, xanthine dehydrogenase, nitrate reductase, formate dehydrogenase, and nitrogenase (77). In humans, sulfite oxidase, xanthine dehydrogenase, and aldehyde oxidase have all been identified as molybdoenzymes, and the absence of these molybdoenzymes, particularly sulfite oxidase, results in severe pathological conditions (37, 57). This medical concern coupled with the fact that nitrogen cycling, which is principally mediated by nitrogen assimilation in plants, requires the participation of molybdoenzymes, has spurred interest in characterizing the steps involved in the successful incorporation of molybdenum into the appropriate apo-molybdoenzymes.

From structural studies, it has been determined that molybdenum exists in molybdoenzymes in the form of a pterin-containing molybdenum cofactor (MoCo), with the exception of nitrogenase which has an iron-molybdenum cofactor (FeMoCo) (1, 31, 57). Other studies have described mutations which result in the pleiotropic loss of molybdoenzyme function (31). One set of these mutants, defined as *chl* mutants in the bacterium *Escherichia coli*, were isolated as chlorate resistant mutants and a major fraction of these were found to be defective in all molybdoenzymes, including nitrate reductase (18, 22, 23, 72).

A subset of these *chl* mutants, designated as *chlD*, exhibited a pleiotropic loss of molybdoenzymes that could be suppressed by molybdate supplementation of the growth medium (14, 22, 29, 48, 61, 67, 71). Further characterization of the *chlD* mutants indicated that the genes in this location comprise the molybdate-specific transporter (now called *modABC*, 69), with *modA* encoding a molybdate-specific periplasmic binding protein, *modB* encoding an integral membrane channel-forming protein, and *modC* encoding an ATP binding energizer protein (33, 47, 58, 60, 75). This molybdate-specific transporter closely resembles the established ATP-binding cassette (ABC) transporter motif (3, 30). Moreover, similar high-affinity molybdate transporters have been described for *Azotobacter vinelandii*, *Rhodobacter capsulatus*, and *Haemophilus influenzae* (20, 43, 76).

The fact that molybdate supplementation of the growth medium restores molybdoenzyme activity in *E. coli mod* mutant strains suggests that molybdate is capable of entering the cell through alternate routes, and preliminary experiments indicate that under high molybdate concentration conditions, molybdate can be transported by the sulfate-specific transporter as well as through other nonspecific anion transporters (41, 61). The molybdate uptake kinetics were also analyzed in *mod* mutant strains, and as expected, *mod* mutants exhibit much lower rates of molybdate transport as compared to *mod*$^+$ strains (14, 29).

Having shown that the *modABCD* genes code for a molybdate-specific transporter, studies concerned with the regulation of expression of the *modABCD* operon in *E. coli* were conducted (24, 48, 58, 61), and it was determined that expression of this operon is

regulated by the intracellular concentration of molybdate. Specifically, high levels of intracellular molybdate resulted in reduction of transcription of the *modABCD* operon, which implies that *E. coli* has a molybdate-dependent repressor which regulates expression of the *modABCD* operon.

Isolation of an *E. coli* strain (strain SE1811) that did not exhibit the classical repression of the *modABCD* operon upon molybdate-supplementation of the growth medium, afforded a starting point for the study of the molybdate-dependent repression of the *modABCD* operon. Determination of the location of the mutation in strain SE1811 led to identification and subsequent characterization of the putative molybdate-dependent repressor (ModE) of the *modABCD* operon. This study serves as an in-depth examination of the ModE and its interaction with the *modABCD* operon, which will ultimately further the understanding of the production of the molybdenum cofactor in *E. coli* and possibly other organisms as well.

LITERATURE REVIEW

One of the hallmark features of many bacteria is the metabolic diversity of their energy producing systems, and the bacterium *Escherichia coli* is no exception. *E. coli* a facultative anaerobe, is capable of switching among its respiratory pathways depending upon the availability of terminal electron acceptors. A hierarchy of electron acceptors exists in *E. coli* as determined by their oxidation reduction potential. Given its large positive redox potential (+ 0.82 V) dioxygen serves as the most efficient electron sink followed by a series of acceptors with incrementally smaller positive redox potentials. When *E. coli* is undergoing aerobic respiration, the proteins contributing to other less energetically favorable pathways of respiration are not synthesized. However in the absence of dioxygen, the components of other respiratory pathways may be produced. Depending upon the availability of alternative electron acceptors, nitrate respiratory components may be synthesized followed by those necessary for the utilization of other alternative electron acceptors such as dimethyl sulfoxide, trimethylamine N-oxide and fumarate-utilizing respiratory pathways (25).

In *E. coli* a number of proteins which transfer electrons from a donor to the final acceptor are molybdoenzymes. These include nitrate reductase, dimethly sulfoxide reductase, and trimethylamine-N-oxide reductase and are molybdoenzymes. All of these proteins contain the molybdenum at the site of electron transfer (77). Structural studies

have shown that the molybdenum in these enzymes is present as a molybdopterin guanine dinucleotide (MGD) moiety (4 5, 35 57)

The identification of the molybdenum cofactor structure as MGD and the determination of the pathway by which it is synthesized resulted from the work of many individuals presented in a number of studies. One early revelation in this inquiry resulted from the identification of a series of pleiotropic mutations in *Aspergillus nidulans* each of which caused a deficiency in the molybdoenzymes xanthine dehydrogenase and nitrate reductase (53) This mutual finding led investigators to believe that there is a molybdenum cofactor common to molybdoenzymes. It was subsequently demonstrated that the inactive apoprotein nitrate reductase produced by a pleiotropic *nit 1* mutant of *Neurospora crassa* could be reconstituted by the addition of deactured preparations of purified molybdoenzymes from animal, plant fungal, or bacterial sources (34) This finding further indicated that molybdoenzymes have a dissociable molybdenum moiety and also showed that this cofactor was common to a variety of organisms

Since the native molybdopterin cofactor proved extremely labile, initial attempts to determine the structure of the active molybdenum cofactor involved derivatization of the cofactor using vigorous oxidation at 100° C in the presence of iodine gas and potassium iodide (KI) creating derivative Form A or KI oxidation in the presence of air resulting in derivative Form B (35). Analysis of these derivatives led to the identification of the molybdopterin (MPT) structure for the molybdenum cofactor Later structural studies showed that dinucleotides such as guanine dinucleotide adenine dinucleotide cytosine dinucleotide, and hypoxanthine dinucleotide can also be associated with the cofactor in the

native molybdoenzymes (36). In *E. coli* and *S. typhimurium* LT2 DMSO reductases contain the molybdopterin guanine dinucleotide form of the molybdenum cofactor (57).

Determination of the structure of the active form of the molybdenum cofactor facilitated studies of the biosynthetic pathway by which the molybdenum cofactor is produced. A great deal of this work was accomplished in *E. coli* since a number of pleiotropic molybdoenzyme mutants had been isolated as chlorate-resistant strains (18, 22, 23, 72). Chlorate, a structural analog of nitrate, can be reduced by nitrate reductase to chlorite, and in solution, chlorite exists in the form hypochlorite which is toxic to the cell. The chlorate-resistant mutants that have been isolated can be classified as those which are only deficient in nitrate reductase due to a mutation in the nitrate reductase structural genes (*chlC* renamed *narGHJI*, 4, 18, 31, 72, 73) and those which are pleiotropic (defined as *chlA*, renamed *moaA*; *chlB*, renamed *moaB*; *chlD*, renamed *moaD*; *chlE*, renamed *moaE*; *chlG*, renamed *moaG*; 69). These pleiotropic mutants are deficient in nitrate reductase and presumably all of the other molybdoenzymes in *E. coli* (formate dehydrogenase-N, formate dehydrogenase-H, DMSO/TMAO reductase, and biotin-sulfoxide reductase) because of a failure to synthesize and incorporate the molybdenum cofactor into the appropriate apoprotein. However, it has been found that the *moaG* mutants do contain functional molybdoenzyme formate dehydrogenase-H (73).

From the characterization of these various pleiotropic chlorate-resistant *E. coli* mutants, the biosynthetic pathway for MGD synthesis in *E. coli* was established and is illustrated in Fig. 1 (57). It was found that the biosynthesis of MGD begins with the conversion of guanosine or a guanosine derivative by the *moa* encoded proteins to a

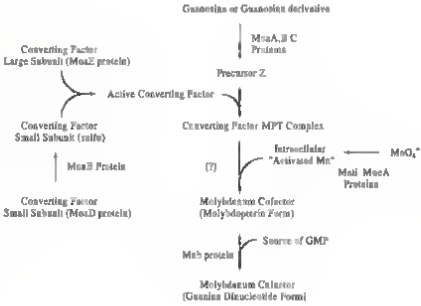


Fig. 1 Steps involved in the biosynthesis of the organic portion of the molybdenum cofactor in *E. coli* (modified from Rajagopalan and Johnson, Ref. 57)

precursor compound, termed precursor Z. Precursor Z joins with active converting factor to produce the converting factor-MPT complex. The active converting factor responsible for the conversion of precursor Z to the converting factor-MPT complex is capable of mediating the conversion only after the small unit of the converting factor (MoaD) protein is first activated by the MoeB protein so that it can then form a complex with the large subunit of the converting factor (MoaE protein), thereby producing the active converting factor. At this point, it is believed that activated molybdenum combines with the molybdopterin molecule. In the presence of guanosine triphosphate, the MobA protein then guanylylates the molybdopterin form of the molybdenum cofactor to the guanine dinucleotide form of the cofactor.

Molybdate Transport

As has already been indicated, a number of pleiotropic *E. coli* chlorate-resistant mutants exist, and one type in particular, referred to as *mod* mutants, is deficient in the uptake of molybdate in the absence of molybdate supplementation of the growth medium (18, 22, 23, 71). Sequencing of the *mod* genes, located in the 17 minute region of the chromosome, resulted in the identification of a four gene operon designated *modABCD*. modA codes for a 257 amino acid molybdate-specific periplasmic binding protein, *modB* codes for a 229 amino acid integral membrane channel-forming protein, *modC* codes for a 352 amino acid transport energizer protein, and *modD* codes for a 231 amino acid putative outer membrane protein which may function as a porin (33, 47, 58, 60, 75). It should be noted that the molybdate-specific transporter encoded by the *modABCD* operon resembles the ATP binding cassette transporter motif in which there is a substrate-specific

periplasmic-binding protein (3 30) an integral membrane channel-forming protein(s), and an ATP-binding protein which is thought to couple the hydrolysis of ATP to transport

Moreover, when molybdate uptake kinetics were investigated in an *E coli mod* mutant strain, the rate of uptake was substantially lower than that seen in the wild type strain, and this transport was shown to be an energy requiring process (14 29) Other presumptive *mod* mutant strains transported molybdate as efficiently as the wild type strain, but these strains were not capable of retaining the imported molybdate, as evidenced by a rapid loss of accumulated molybdate when chased with unlabeled molybdate (14) This result implies that the mutation in that second group of mutants is not located in one of the genes coding for the molybdate-specific transporter it apparently is located in a gene necessary for the incorporation of the molybdenum into the cellular material

Homologs of the *E coli* molybdate-specific transport proteins have also been identified in other facultative anaerobes such as *H Influenzae* Rd as well as the nitrogen-fixing bacteria *A vinelandii* and *R capsulatus* (20 43 76) In the case of the *H influenzae* Rd Mod proteins, a 49% identity and 58% similarity for ModA was determined while a 65% identity and 73% similarity for ModB and a 53 % identity and 66% similarity for ModC were found in relation to the respective *E coli* proteins The *A vinelandii* homologs show 27% identity and 38% similarity compared to *E coli* ModA, 31% identity and 47% similarity for ModB and 44% identity and 58% similarity for ModC The *R capsulatus* molybdate transport protein homologs have 29% identity and 40% similarity

for ModA, 29% identity and 45% similarity for ModB and 39% identity and 55% similarity for ModC compared to the *E. coli* proteins.

Aside from the sequence similarity implicating the *A. vinelandii* and *R. capsulatus* homologs involvement in molybdate-specific uptake, there are also genetic studies which suggest that these proteins do indeed constitute the molybdate-specific transporter in the respective organism. One such study (50) demonstrated that mutations in the *modB* and *modC* genes of *A. vinelandii* allowed for expression of the alternate nitrogenase (heterometal-free nitrogenase) in the presence of 0.5 μM sodium molybdate. Since it has previously been shown that expression of the alternate nitrogenase is repressed in the presence of molybdate in the wild type (43), it may be concluded that these mutations prevent molybdate-specific transport. Furthermore, mutations in *modA*, *modB* or *modC* of *R. capsulatus* resulted in a loss of activity for the molybdenum-containing nitrogenase as well as derepression of the transcription of the genes encoding the heterometal-free alternative nitrogenase, which again indicates a loss of molybdate-specific transport by these mutants (76). In both of these organisms, increasing the concentration of the molybdenum in the medium resulted in repression of transcription of the alternate nitrogenase genes.

The fact that mutations in genes encoding the molybdate-specific transporter proteins can be suppressed by the addition of sufficiently high concentrations of molybdate to the growth medium suggests that alternate routes for molybdate transport exist (41, 61). Furthermore, there is evidence that molybdate is capable of being transported through

the sulfate/thiosulfate and selenate transport systems (41, 61), which is not surprising given the similarity in charge and structure among molybdate, sulfate, and selenate.

Regulation of the *modABCD* Operon

Expression of the *modABCD* operon has been investigated in a variety of genetic backgrounds by means of monitoring β-galactosidase activities in strains harboring *φ*(*modA - lacZ*) (58, 60, 61). From these experiments, it was found that *modA - lacZ* expression was high under conditions of low intracellular molybdate as in the case of *mod* mutants grown in medium lacking molybdate. However, when there is a high enough intracellular molybdate concentration to support nitrate reductase or formate hydrogenlyase activity as is the case in a molybdate transport competent strain or a *mod*$^+$ strain grown in molybdate supplemented medium, *modA - lacZ* expression is very low. Since molybdate availability seemed to effect expression of the *modABCD* operon, the possible role of MGD synthesis in *mod* expression was also explored, and it was found that mutations in the *moa*, *mob*, or *moe* operons did not appreciably change expression of the *modABCD* operon. However, *modA - lacZ* expression was increased approximately two-fold when the cells were grown under aerobic conditions or when cultured anaerobically in a strain containing an *fnr* mutation (60, 61). Inspection of the DNA sequence upstream of *modA* transcription start site did not reveal any putative FNR protein binding motif; consequently, this increase in expression of the *modABCD* operon in an *fnr* background is most likely a physiological effect of the *fnr* mutation on other operons like *narGHJI*.

A study by Rech et al. (58) examined the effects of molybdate, nitrate and oxygen on *modABCD* expression, and again it was found that molybdate limitation enhanced

modABCD expression, whereas nitrate and oxygen did not significantly modify expression levels. This study also evaluated the DNA sequence in the *modA* operator/promoter region which might be involved the molybdate-dependent regulation of the *modABCD* operon. It was determined by mutation analysis that a CATAA sequence located at position +2 to +6 in relation to the *modA* transcription start site is involved in binding the molybdate-dependent regulator; however a similar CATTAA sequence located at position +12 to +17 was not required for regulation.

Further analysis of the *modA* operator/promoter DNA revealed that there is an eight base pair (bp) inverted repeat sequence ('TAACGTTA') spanning the +4 to +11 region. This inverted repeat may serve as the actual recognition sequence for the molybdate dependent regulator of the *modABCD* operon since the inverted repeat sequence overlaps the +2 to +6 sequence that had been implicated in regulator protein binding. Inverted repeat sequences have been shown to constitute the binding sites for a number of repressor proteins such as the MetJ repressor protein and the Trp repressor (40, 54, 55).

Although the molybdate-dependent repression of *modABCD* expression has been reported by several investigators, the causative agent of this repression had not been determined. This study identifies this molybdate dependent repressor designated ModE, and also locates the recognition sequences in the *modA* operator/promoter DNA required for binding the repressor. The interaction between the repressor and its target DNA is examined under a variety of conditions.

MATERIALS AND METHODS

Materials

Biochemicals were purchased from Sigma Chemical Co (St. Louis, MO) Inorganic and organic chemicals were obtained from Fisher Scientific Co and were analytical or molecular biological grade Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs, Inc (Beverly MA) or Promega (Madison, WI) S30 cell extract was purchased from Promega (Madison, WI) Sequenase 2 0 was obtained from United States Biochemical Corporation (Cleveland OH)

Bacterial Strains

Bacterial strains used in this study are presented in Table 1 All bacterial strains used are derivatives of *Escherichia coli* K-12

Media

Bacterial cultures were routinely grown in Luria Broth (LB) which contains 1% trypticase peptone (BBL, Cockeysville MD), 0.5% yeast extract (BBL) and 0 5% NaCl. When necessary, the LB was supplemented with 0 3% glucose (LBG) and 1 mM sodium molybdate. Lactose-MacConkey agar was prepared by adding filter-sterilized lactose

Table 1 Bacterial strains and phages used in this study

Strain or Phage	Genotype	Source and/or reference
Strains		
BW545	*Δ(lacU)169 rpsL*	Laboratory collection
MC4100	*araD139 Δ(argF lacU)205 rpsL150 relA1 flbB5301 deoC1 ptsF25*	CGSC 6152
ECL818	*srlA2 zjj::*Tn*10*	E. Lin
RK4353	MC4100 *gyrA219 nov-9*	V. Stewart
RKS2718	RK4353 *narL215::*Tn*10*	V. Stewart
VJS720	*chlD247::*Tn*10* (*modA247*)	V. Stewart
VJS1778	RK4353 *mae-251::*Tn*10d*(Tc)	V. Stewart
VJS1780	RK4353 *mob-252::*Tn*10d*(Tc)	V. Stewart
VJS1782	RK4353 *mae-254::*Tn*10d*(Tc)	V. Stewart
VJS1784	RK4353 *mog-256::*Tn*10d*(Tc)	V. Stewart
BL21λDE3	*hsdS gal* λ[*lacUV5 gene1* (T7)]	F. Studier (74)
SE1325	*cysC43 trl-300::*Tn*10 thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 rpsL31 tsx-33 supE44 modA138::*Tn*5*	Laboratory collection
SE1592	BW545 *modA115*	Laboratory collection
SE1595	BW545 *modC118*	Laboratory collection
SE1602	BW545 *modB125*	Laboratory collection
SE1811	SE2069 *modE1*	This study
SE1910	BW545 (Δ*modE*::km)2	(24)
SE1934	RK4353 (Δ*modF*::km)1	(24)
SE1938	BW545 λRM26	(24)
SE1940	BW545 (Δ*modF*::km)1 λRM26	P1 transduction (SE1938 x SE1934)
SE1942	BW545 (Δ*modE*::km)2 λRM26	P1 transduction (SE1938 x SE1910)
SE1952	BW545 *modA138*::Tn*5* λRM26	P1 transduction (SE1938 x SE1325)
SE2069	BW545 Φ(*modA'::lacZ'*)*102*	Laboratory collection
SE2105	BW545 λRM37	R. Ray
SE2106	SE1910 λRM37	R. Ray
SE2107	BW545 (Δ*modF*::km)1 λRM37	R. Ray
SE2112	BW545 (Δ*modEF*::km)1 λRM37	R. Ray
SE2114	BW545 *modB*::Tn*10* λRM37	R. Ray
SE2116	SE2106 *modB*::Tn*10*	R. Ray
SE2118	SE2107 *modB*::Tn*10*	R. Ray
SE4110	MC4100 (Δ*modE*::km)*3*	This study

Table 1 Cont.

Strain or Phage	Genotype	
Phages		
ΔRZ5	*bla*$^{+}$*lacZ*$^{+}$*lacY*$^{+}$	Laboratory collection
ΔRM26	ΔRZ5(Φ(*madE*$^{+}$*lacZ*$^{+}$)[	(24)
ΔSE1	ΔRZ5[Φ(*madA* *lacZ*$^{+}$)]	Laboratory collection
ΔRM37	ΔRZ5[Φ(*madF*$^{+}$*lacZ*$^{+}$)]	R. Ray
ΔPC50	ΔRS45(*bla* *lacZ*$_{oc}$) [Φ(*tnerG*	R. P. Gunsalus

(final concentration 1%) to MacConkey agar base (Difco Detroit, MI) after autoclaving Ampicillin (100 μg/ml) kanamycin (50 μg/ml) tetracycline (15 μg/mL), chloramphenicol (15 μg/ml) and X-gal (40 μg/ml) were included in the media as needed.

Enzyme Activities and Culture Conditions

β-Galactosidase activities Standing overnight LB cultures were used to inoculate (5% v/v) fresh medium in 13x100 mm screw cap tubes The cultures in filled tubes were incubated at 37°C until late-exponential or early stationary phase had been attained (4 to 6 h) The β-galactosidase activity of Sodium-dodecyl-sulfate (SDS)-chloroform permeabilized cells was assayed by measuring the rate of hydrolysis of o-nitrophenyl-β D-galactopyranoside (ONPG) as per Miller (49) The activities are expressed as nanomoles of o-nitrophenol produced per minute per milligram of protein where a value of 350 μg protein/ml was used as the protein content of 1 ml of cells at an optical density (O D) of 1 0 unit in a Spectronic 710 (Rochester, NY) spectrophotometer

FHL activities. Formate hydrogen lyase activity was qualitatively determined as previously described (61)

Genetic Experiments

All genetic experiments were conducted as per standard protocols (49)

Molecular Biology Experiments

Plasmid constructions. The plasmids employed in this study are listed in Table 2 Standard published procedures (45) or DNA modifying enzyme suppliers' recommendations were used for plasmid constructions and manipulations

Table 2. Plasmids used in this study

Plasmid	Relevent Genotype	[illegible]
pACYC184	Cam^r Tet^r	(11)
pBR322	Amp^r Tet^r	(8)
pTrc99A	*Ptrc lacI*^{q} Amp^r	(2)
pT7 7	pT7 *lacZ*' Amp^r	J. Maupin
pUC19	Amp^r	(78)
pUC4K	*lacZ* Kan^r Amp	Pharmacia
pZ1918	*lacZ* Amp^r	H. Schweizer (66)
pMAK705	*lacZ rep*(ts) *cam*^{r}	(26)
pFGH15	pACYC184 (*modABC DEF*) *gal*	(47)
pSE1004	pBR322 (*modABCDE*)	(41)
pSE1009	pUC19 (*modABC*)	(41)
pAG1	pTrc99A (*modE*$^+$)	This study
pAG1-A76V	pTrc99A (*modE76*)	This study
pAG1-T125I	pTrc99A (*modE125*)	This study
pAG1-G133D	pTrc99A (*modE133*)	This study
pAG1-Q216*	pTrc99A (*modE216*)	This study
pRM1	pUC19 (*modA*$^+$)	(24)
pRM9	pBR322 [*modE*-km (*modABCD*)]	(24)
pRM10	pMAK705 (*modE* km)	(24)
pRM12	pUC19 (*modEF*) *galE*	(24)
pRM13	pBR322 (*modEF*) *galG*	(24)
pRM14	pBR322 {Φ(*modE*' *lacZ*)}	This study
pRM16	pBR322 (*modE*' *modF* km)	(24)
pRM17	pMAK705 ['*modE* Φ(*modF* km)]	(24)
pRM22	pT7 7 (*modEF*)	(24)
pRM23	pT7 7 (*modF*')	(24)
pRM25	pBR322 (Δ *Hin*dIII *Ava*I fragment) (*modEF*) *galE*	(24)
pRM26	pBR322 [Φ(*modE*' *lacZ*) *modF*'[	(24)
pAM4	pUC19 (247 to +25 *modA* operator/ promoter)	This study
pAM5	pUC19 (-85 to +25 *modA* operator/promoter)	This study
pAM6	pUC19 (-79 to +25 *modA* operator/promoter)	This study
pAM7	pUC19 (55 to +25 *modA* operator/ promoter)	This study
pAM8	pUC19 (-30 to +25 *modA* operator/ promoter)	This study
pAM9	pUC19 (247 to 55 *modA* operator/ promoter)	This study
pAM10	pUC19 (-11 to +25 *modA* operator/promoter)	This study

Table 2. Contd.

Plasmid	Relevant Genotype	Source or reference
pAM13	pUC19 (*mod* DNA –15 to *Acc*I in *modA*)	This study
pAM15	pUC19 [247 to +25 Δ(+4 to +11) *modA* operator/promoter]	This study
pAM16	pUC19 (243 to +20 *modA* operator/promoter	This study
pAM17	pUC19 (–17 to +6 *modA* operator/promoter)	This study
pAM18	pUC19 (–17 to 1 +5 to +15 *modA* operator/promoter	This study
pAM19	pUC19 [17 to +15 Δ(–6 to +9) *modA* operator/promoter	This study
plagG	pGEM3ZF+ [(*modE*$^+$), *Haemophilus influenzae*]	Apicella
plagH	pGEM3ZF+ [(*modA*$^+$), *Haemophilus influenzae*]	Apicella

A Φ(*modE* - *lacZ*) plasmid was constructed by first removing a 2.6-kb *KpnI*-*HindIII* fragment from plasmid pFGH15 containing the *mod EF galE* genes and ligating it into the *KpnI*-*HindIII* sites of plasmid pUC19 to yield plasmid pRM11. Since the 5' region of the *modE* gene was not present in plasmid pRM11, a 0.6-kb *KpnI* fragment from plasmid pFGH15 containing the 5' region of the *modE* gene was inserted in frame to construct plasmid pRM12. The *modEF galE* DNA was then moved to plasmid vector pBR322 by ligating a 3.2-kb *EcoRI*-*HindIII* fragment from plasmid pRM12 into the corresponding sites in plasmid pBR322, resulting in plasmid pRM13. Plasmid pRM13 proved to be unstable; however, the removal of the *tet* gene in the vector DNA as a *HindIII*-*AvaI* fragment from plasmid pRM13 to give plasmid pRM25 resolved the instability problem. A 3.2-kb *SmaI* fragment encoding the *lacZ* gene from plasmid pZ1918 (66) was then ligated into the *AflII* site (in *modE* gene) of plasmid pRM13 which had previously been modified using the klenow fragment of DNA polymerase I (Klenow), thereby creating plasmid pRM26. Plasmid pRM26 produced β-galactosidase activity from the *modE* promoter. Subsequent transfer of the Φ(*modE* - *lacZ*) into the chromosome of strain BW545 was accomplished by first recombining in vivo the *bla* and *modE* - *lacZ* from plasmid pRM26 with λRZ5 to produce λRM26. Strain BW545 was then transduced with phage λRM26.

In the course of this study, two different *modE* expression plasmids were constructed. Plasmid pAG1, used for the production of ModE mutants, was created by ligating a Klenow-modified 870-bp *EspI* fragment containing the promoterless *modE* gene from plasmid pFGH15 into the *SmaI* site of the *ptac*-based expression vector

pTrc99A. The second *modE* expression plasmid, pRM22, used for purification of ModE, was constructed by ligating an *Eco*RI–*Hin*dIII fragment which contained the *modEF* genes from plasmid pRM12 into the *Eco*RI–*Hin*dIII sites of plasmid pT7-7.

The *modAB*-containing plasmid, which was used in the in vitro coupled transcription-translation experiments, resulted from the ligation of a 1.7-kb *Msc*I fragment from plasmid pSE1009 into the *Sma*I site of plasmid pUC19, yielding plasmid pRM1. Plasmid pRM1 carries the entire *modA* gene but a truncated *modB* gene (modB lacks the C-terminal ten amino acids).

A battery of *modA*-operator/promoter DNA-containing plasmids were constructed for the purpose of in vivo ModE titration experiments. One such plasmid, pAM4, was produced by ligating a *Kpn*I–*Bst*EII fragment from plasmid pSE1009 (the *Bst*EII end was modified using Klenow enzyme) which covered the −247 to +25 sequence of the *modA*-operator/promoter region into the *Kpn*I–*Hin*cII sites of plasmid pUC19. The plasmid pAM4 deletion-derivative plasmids pAM5 and pAM6 were obtained by nuclease Bal-31 treatment of *Kpn*I-digested plasmid pAM4 followed by religation. The extent of the deletions in plasmids pAM5 and pAM6 was determined by sequencing the DNA. Another derivative of plasmid pAM4, plasmid pAM7, was created by removal of an *Eco*NI–*Eco*RI fragment (-247 to −56 of *modA*-operator/promoter region) from plasmid pAM4 and subsequent religation after the ends had been filled in with Klenow. Plasmid pAM8 was constructed by removal of a 217-bp *Apo*I fragment (-247 to -31 of *modA*-operator/promoter region) from plasmid pAM4, and plasmid pAM9 was achieved by ligating a *Kpn*I–*Apo*I fragment (-247 to −55 of *modA*-operator/promoter region) from plasmid

pAM4 into the *KpnI-ApoI* sites of plasmid pUC19. Plasmid pAM10 was produced by digesting plasmid pAM8 with *ApoI* followed by nuclease Bal-31 treatment and religation. Again, the extent of the deletion was determined through sequencing the DNA.

Construction of plasmid pAM13 relied on polymerase chain reaction (PCR) generation of a 648-bp fragment using the following primers synthesized by National Biosciences, Inc. (NBI, Plymouth, MN). Primer #1, 5' AAGGATCCGTTATATTGTCGCCTAC 3', which contains an engineered *BamHI* site and is complementary to the -16 to +2 sequence of the *modA* operator/promoter and Primer #2, 5' GCTGGCAACTGCGTC 3', which is complementary to sequence located in the 3' end of *modA*. The 648-bp fragment was digested with *BamHI* and *AccI* to give a 634-bp fragment which was ligated into the *BamHI/AccI* sites of plasmid pUC19, thereby yielding plasmid pAM13. Plasmid pAM15 was obtained as a result of mung bean nuclease treatment and religation of *PstI*406-digested plasmid pAM4. Plasmid pAM16 resulted from mung bean nuclease treatment of the *BstEII* end of a *KpnI, BstEII* fragment from plasmid pSE1009 which was then ligated into the *KpnI-HincII* sites of plasmid pUC19. Plasmids pAM17, pAM18, and pAM19 were constructed by ligating the oligomers which contained various portions of *modA*-operator/promoter sequence into vector plasmid pUC19. These DNA oligomers were synthesized by NBI. Complementary oligomers were annealed, polynucleotide kinase-treated and digested with *BamHI* prior to ligating into the *BamHI-SmaI* sites of plasmid pUC19. The *mod*-insert DNA in plasmid pAM17 has the following 36-bp sequence 5' CGCAATCGTTATAT TGTCGCCTACATAAGATCCCG 3', while plasmid pAM18's insert has a 45-bp sequence, 5' CGGAATCGTTATATTGTCGCCTTGGAACGTTAC

ATTGGATCCCG 3 and plasmid pAM19 contains the following 31-bp sequence 5 CGGAATCGTTATATTGTTACATTGGATCCCG 3 insert.

Hydroxylamine mutagenesis of plasmid DNA. Hydroxylamine mutagenesis of plasmid DNA was carried out essentially as described by Davis et al. (15) with minor modifications. A reaction mixture containing 7 5 µg plasmid pAG1 DNA, 40 µl of phosphate EDTA buffer (0 5 M K_2PO_4, pH 6 5 5mM EDTA), 80 µl of freshly prepared hydroxylamine-hydrochloride solution, pH 6 0 (0 35 g of NH_2OH-HCl, 0 56 ml of 4M NaOH, 4 44 ml distilled H O) and 50 µl deionized H_2O was incubated at 37°C for 18 h. Following the incubation period, hydroxylamine was removed by three successive 1 1 phenol extractions and a 1 1 chloroform extraction. At this point, the plasmid DNA was ethanol precipitated, and the dissolved DNA was used to transform competent cells of strain RK4353 Total plasmid DNA isolated from these transformants was then transformed into competent cells of strain SE1§11 for identification of mutant ModE containing plasmids. The resultant transformants were plated on lactose-MacConkey agar with and without 1mM sodium molybdate. White colonies were picked from media lacking molybdate, and red colonies were picked from the media supplemented with molybdate The levels of β-galactosidase activity were determined for the SE1§11 cells containing the mutant plasmids to confirm the presumptive phenotypes. The entire *modE* sequence was then determined for each selected mutant

DNA Sequencing experiments. All DNA sequences generated in the course of this study were obtained using the Sanger dideoxy method and appropriate primers and plasmids which had been isolated by alkaline lysis procedure and purified by cesium

chloride density-gradient centrifugation (28 62) For sequencing mutant *modE* genes, the primers used were synthesized by the DNA Synthesis Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida based on the sequence of the wild type *modE* DNA. The DNA sequences were analyzed by using computer software programs GCG (6..13 16) and Genepro (Riverside Scientific, Seattle, WA)

Biochemical Characterization of ModE

Expression and purification of ModE. For isolation of ModE protein, a 1L LB culture of strain BL21λDE3(pRM22) was incubated at 37°C with vigorous shaking (250 rpm) until an optical density of 1 0 was reached. Isopropyl-β-D-thiogalacto-pyranoside (IPTG) was then added to a final concentration of 0 5 mM to induce phage T7 RNA polymerase and high level expression of *modE* After incubation for an additional two h., the cells were harvested by centrifugation at 8 300 x g for 10 min. and were resuspended in 8 ml of extraction buffer [50 mM Tris-HCl, pH 7 5 0 5 mM EDTA, 0.5 % glycerol, 1mM dithiothreitol (DTT)] This cell suspension was passed twice through a French pressure cell (20,000 lb/in²) The broken cell suspension was centrifuged at 100 000 x g for 60 min. to remove cellular debris. The resultant supernatant was loaded onto a 10 ml Q Sepharose fast-flow column (Pharmacia) that had been equilibrated with 50 mM Tris-HCl, pH 8 0 Progress of the protein sample through the column was monitored using a chart recorder connected to a UV light (280 nm) absorbance detector After application of the protein sample to the Q sepharose column, the column was washed with 50 mM Tris-HCl, pH 8 0 until a baseline on the UV absorbance trace had been established The column was subsequently washed with Tris-HCl, pH 8 0 buffer containing 0 1 M NaCl. The

column was washed with this buffer until protein no longer eluted from the column as indicated by a return to the baseline on the UV absorbance trace. The column was then washed successively with Tris-HCl, pH 8.0 containing 0.2 M, 0.3 M, 0.4 M, 0.5 M, and 1.0 M NaCl. Fractions containing the ModE protein eluted with 0.2 M NaCl. The buffer of the ModE-containing protein solution was exchanged to 10 mM phosphate buffer, pH 7 by using an EconoPac-10 DG desalting column (BioRad Laboratories, Hercules, CA) prior to loading the protein on to a 5 ml heparin column (HiTrap; Pharmacia) that had been equilibrated with 10 mM phosphate buffer, pH 7. Bound proteins were stripped from the heparin column by successive washes with 10 mM phosphate buffer, pH 7.0 containing 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, and 1.0 M NaCl. The elution procedure proceeded as indicated above for the Q sepharose chromatography. ModE-containing protein fractions eluted at a salt concentration of 0.2 M. At this point, the ModE-containing protein sample was concentrated using a Centricell-20 spin cartridge (Polysciences, Warrington, PA). The buffer of the ModE solution was exchanged with 25 mM histidine-HCl buffer, pH 6.2 and the protein was applied to a 10-ml polybuffer exchanger 94 column (Pharmacia) equilibrated with 25 mM histidine-HCl buffer, pH 6.2. The column was washed with polybuffer 74, pH 4.0, and the ModE protein was eluted when the pH of the effluent reached approximately 4.5. The ModE protein was stored in 10 mM phosphate buffer–10% glycerol, pH 7.0 at a final protein concentration of 0.42 mg/ml. The entire purification process was conducted at 4 °C. Since the intrinsic properties of the ModE protein did not lend itself to conventional detection by UV light at 280 nm or detection by the Bradford assay (9), ModE protein was monitored by the

bicinchoninic acid protein determination assay (70) as well as by electrophoresis in (SDS) polyacrylamide gels. N-terminal amino acid sequencing (performed at the Interdisciplinary Center for Biotechnology Research Protein Chemistry Core Laboratory at the University of Florida) was used to confirm the identity of the purified protein as ModE. Matrix-assisted-desorption time-of-flight spectrometric determination of the molecular weight of purified ModE protein was kindly performed by Dr. Preston.

In vitro coupled transcription-translation experiment. In vitro transcription-translation experiments using S30 extract and L-[^{35}S]methionine to monitor the expression of *modA* and *modB* from plasmid pRM1 in the presence and absence of ModE and 1 mM sodium molybdate were conducted as suggested by Promega. Three µg of plasmid pRM1 and when appropriate, 40 pmol of ModE and 1 mM sodium molybdate were included in the S30 reaction mixtures. The ^{35}S-labeled proteins were separated by electrophoresis through a SDS-15% polyacrylamide gel. Following completion of the electrophoresis, the resulting gel was transferred to Whatman 3mm paper and was dried under vacuum. X-ray film (Fuji RX) was then applied to the gel for autoradiographic visualization of the labeled proteins. Quantification of labeled proteins was accomplished by using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Mobility shift experiments. DNA-mobility shift experiments were performed as described by Fried and Crothers (21) with modification. These experiments used a binding reaction buffer consisting of 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 5% glycerol. The reaction mixture contained the binding reaction buffer, DNA, and protein in a final volume of 10 µl. The reaction mixtures were incubated at

37°C for 30 min. prior to loading onto 5%-polyacrylamide gels in Tris-borate-EDTA buffer that had been pewon at 100 V at room temperature for approximately 60 min (51). During the electrophoresis, tank buffer was continuously circulated using a Minipuls II pump (Gilson). The migration patterns of the binding reactions in the resulting gels were visualized by autoradiography and various bands in the gels were also quantified using a phosphorimager.

Experiments designed to ascertain the apparent dissociation constant (K_d) for the binding of ModE to its target DNA featured a 43-bp oligonucleotide synthesized by NBI which spans the -18 to +25 region of the *mod*-operator/promoter sequence. For the DNA-mobility shift experiments, the 43-bp oligonucleotide was end labeled using kinase to phosphorylate the 5' ends with γ [^{32}P] from γ [^{32}P]–deoxyadenosine triphosphate (dATP). Unincorporated label was removed by passing the labeled oligonucleotide through a G-25 microspin column (Pharmacia). The reaction mixtures prepared for these experiments contained 0.1 pmol of the 43-bp labeled oligonucleotide as well as 0, 1, 5, 10, 25, 50, 100, 250, 500, and 1000 fold excess of ModE. When appropriate, sodium molybdate at a final concentration of 10 μM or 1 mM was also included in the reaction mixtures. Experiments which had sodium molybdate in the reaction mixtures also contained sodium molybdate in the gels and electrophoresis buffers at the same concentration as that present in the binding reactions. In some experiments, 10 μM sodium molybdate was replaced with sodium tungstate, sodium sulfate or sodium ortho-vanadate at the same concentration as in the reaction mixtures, gels and tank buffers.

For the DNA mobility shift experiments designed to determine the concentration of molybdate required for half-maximal binding of ModE the binding reactions included different concentrations of sodium molybdate ranging from 1 pM to 1 mM as well as 0.1 pmol 43 bp oligomer and 50-fold excess of ModE. Two polyacrylamide gels were run for this experiment, one with 1 pM sodium molybdate present in the gel and tank buffer and a second gel having 1 μM molybdate present. Binding reactions with 1 pM to 1 μM molybdate were electrophoresed through the 1 pM molybdate gel, and binding reactions having 1 μM to 1 mM molybdate were subjected to electrphoresis through the gel containing 1 μM molybdate.

In this study, the measure of the affinity of ModE for its target DNA is reported as an apparent dissociation constant value (K_D value). The dissociation constant is defined as the concentration of protein required for 50% binding to the target DNA. Apparent K_D values describing binding of ModE to DNA were determined by plotting the concentration of ModE present in the binding reaction versus the resultant percent of shifted DNA.

In vitro DNase I footprinting experiments. The DNase I protection experiments were carried out essentially as described previously (42, 56, 65). For these experiments, a 446-bp *Fsp*I *Hind*III fragment from plasmid pAM4 which carries *modA*-operator/promoter DNA spanning from -247 to +25 was labeled using α-^{32}P-dATP, dCTP, dGTP, and dTTP (NEN, Boston, MA) using Klenow to fill in the *Hind*III end. Binding reactions having a total volume of 20 μl were prepared which contained 0.1 pmol of the labeled DNA, 0, 1, 10, 100, or 1000-fold excess ModE, 1 mM sodium molybdate, and the binding reaction buffer used in the DNA-mobility shift experiments. The binding reactions were

incubated for 30 min. at 37°C prior to adding 1.5 ng of DNase I (Sigma). The DNase I treatment continued for two minutes before adding 15 µl stop solution (34 mM EDTA, 6.5 M ammonium acetate). Ethanol (110 µl) was then added to precipitate the cleaved DNA fragments. The precipitated DNA was resuspended in 6 µl deionized H_2O and 4 µl Sequenase stop solution (USB) and the samples were electrophoresed through an 8% polyacrylamide TBE-denaturing gel. After drying the gel, autoradiography was used to determine the areas of DNA protected by ModE from DNase I cleavage.

Determination of the molecular weight of the ModE-43 bp-DNA complex using native gel electrophoresis. In an effort to determine the stoichiometry of the ModE-DNA association, binding reactions containing 0.1 pmol of a 43-bp oligomer (-18 to +25 region) and 250-fold excess ModE were prepared as described for the DNA mobility shift experiments. The binding reaction samples were then electrophoresed through a 5.0%, 6.0%, 7.0% or 8.0%-polyacrylamide TBE nondenaturing gel as described under DNA mobility shift experiments. The differences in the R_f values [migration of the sample (cm)/ migration of the dye front (cm)] for the ModE-DNA complexes after electrophoresis through the different concentration polyacrylamide gels was then compared to values obtained for protein standards. For the determination of the R_f values of the protein standards: 15 µg of lactalbumin (14,200 Da), 20 µg of carbonic anhydrase (29,000), 20 µg of chicken egg albumin (45,000 Da), 15 µg of bovine serum albumin (66,000 Da monomer, 132,000 Da dimer) and 6 µg of urease (240,000 Da dimer and 480,000 Da tetramer) were electrophoresed along with the ModE-DNA complex in various polyacrylamide TBE-nondenaturing gels. A plot of the [$\log(R_f \times 100)$] versus % gel

concentration for the molecular weight standards was then generated, and the slopes derived for each of the standards from this plot were then used to produce a Ferguson plot (19), which relates the negative slope to molecular weight of the protein. The molecular weight of the ModE-43 bp-oligomer complex was determined by extrapolation from the Ferguson plot

RESULTS AND DISCUSSION

Evidence for a Regulator of the *modABCD* Operon

Previous work demonstrated that a mutation resulting in loss of function in any one of the proteins which form the molybdate-specific transporter, ModA, ModB, or ModC will cause a loss of activity for the cell's complement of molybdoenzymes (58, 61). This phenotype, which can be suppressed by molybdate supplementation of the growth medium, suggests that the cells containing these mutations are unable to transport molybdate under conditions of low molybdate concentration in the growth medium (22, 29, 48, 61, 67, 71). Restoration of the activities of molybdoenzymes with the addition of 1 mM molybdate to the growth medium also indicates that a sufficient intracellular concentration of molybdate is generated by alternate routes of molybdate transport other than the molybdate-specific transport system in *E. coli* (41, 61). Although a molybdate-specific transport system has been defined in various organisms, the mechanism for the regulation of the genes coding for the components of the transport machinery is not known.

A general approach for initial investigation of the regulation of genes of interest in *Escherichia coli* is to construct *lacZ* fusions in the appropriate genes and then monitor the resulting β-galactosidase activities under a variety of experimental conditions and in a number of genetic backgrounds. In this case, the genes of interest comprise the

modABCD operon, and *E. coli* strain SE2069, which contains a chromosomal ϕ(*modA'l lacZ*) fusion was used. Since strain SE2069 has its *modA* gene interrupted by the *lacZ* gene fusion, it is functionally a *mod*⁻ mutant strain. Using strain SE2069, it was found that a high level of *modA – lacZ* expression, monitored as β-galactosidase activity, was only achieved in cells grown in LBG, a medium which contains trace amounts of contaminating molybdate (Table 3). Therefore, the fact that high level expression of *modA - lacZ* is only seen in the absence of added molybdate intimates that the *modABCD* operon is subject to repression only when sufficient intracellular levels of molybdate are present. If this is indeed the case, a *mod*⁺ *E. coli* (strain BW545) should transcribe the *modABCD* operon at very low levels. In order to test this possibility, the *modA - lacZ* fusion from strain SE2069 was transferred to phage (λSE1) and incorporated into the λ *att* site of the chromosome.

Low levels of expression of *modA - lacZ* in the wild type *mod*⁺ strain, strain BW545(λSE1), regardless of supplementation of molybdate to the growth medium (Table 3) implies that the functional molybdate-specific transporter brings in sufficient levels of molybdate from the LBG medium to repress the *modABCD* operon. In the presence of a mutation in any of the first three *mod* genes (undefined point mutation in a given *mod* gene), derepressed levels of *modA – lacZ* from λSE1 result only in the absence of molybdate supplementation of the growth medium.

Further information concerning the nature of the molybdate dependent repression of the *modABCD* operon was afforded by complementation studies of a spontaneous

Table 3 Effect of *mod* gene mutation in *mod* operon transcription

Strain	*mod* Genotype	β-galactosidase Activity[a] -Mo	+Mo
SE2069	ϕ(*modA* = *lacZ*)	2 400	250
BW545(λSE1)	*mod*$^+$	20	<10
SE1592(λSE1)	*modA*	2 330	165
SE1595(λSE1)	*modC*	1 980	150
SE1602(λSE1)	*modB*	1 860	60

[a]Cells were grown in LBG medium supplemented with 1 mM sodium molybdate when specified. β-galactosidase activities are expressed as nmoles minute^{-1} milligram of cell protein

mutant of strain of SE2069 designated as strain SE1811. The level of *modA'-lacZ* expression by strain SE1811 remained high even in the presence of 1 mM molybdate, suggesting that this mutation inactivated the gene responsible for the molybdate-dependent repression of the *modABCD* operon (Table 4).

By phage P1-mediated transduction, the mutation which derepressed *modA* expression in strain SE1811 was mapped close to *modA*. The identification of the gene altered in strain SE1811 was accomplished through the introduction of plasmids containing different regions of the *mod* DNA into strain SE1811. As presented in Table 4, the introduction of plasmid pSE1004 which harbors *modABCD* DNA as well as an open reading frame diverging from this operon into strain SE1811 resulted in both the restoration of molybdate transport as indicated by the return of FHL activity in the absence of added molybdate in strain SE1811 as well as the restoration of molybdate-dependent repression of the *modABCD* operon in this strain. Introduction of plasmid pSE1009 which has *modABC* DNA only restored the molybdate-specific transport in strain SE1811 as seen by the presence of FHL activity but did not restore the molybdate-dependent repression of *modA'-lacZ* expression in this strain. As expected both plasmids complemented the *modA* mutation in parent strain SE2069.

It should be noted that there is a disparity in the β-galactosidase activity values produced by strain SE2069 (Tables 3 and 4) which is most likely the result of allelic variations in strain SE2069. The higher value from Table 3 was obtained approximately 2 years prior to the value shown in Table 4, while the value from Table 4 is characteristic of values which are now routinely obtained for strain SE2069.

Table 4 Complementation of the mutation in strain SE1811 for molybdate-dependent repression of the *modABCD* operon

Strain	Relevant Genotype	β-galactosidase Activity[a] -Mo	+Mo	FHL[b]
SE1811	Φ(*modA-lacZ*) *modE*	1,500	1,500	
SE1811(pSE1004)	Φ(*modA lacZ*) *modE* (*modABCDE*)	290	260	+
SE1811(pSE1009)	Φ(*modA-lacZ*) *modE*, (*modABC*)	1,500	1,500	+
SE1811(pAG1)	Φ(*modA lacZ*) *modE* (*modE*)	600	100	
SE2069	Φ(*modA-lacZ*)	1,300	200	-
SE2069(pSE1004)	Φ(*modA-lacZ*), (*modABCDE*)	300	300	+
SE2069(pSE1009)	Φ(*modA-lacZ*), (*modABC*)	300	300	+

[a]Cells were grown in LBG medium. Sodium molybdate concentration was 1 mM. β-galactosidase activities are expressed as nanomoles minute^{-1} milligram of cell protein^{-1}
[b]-, absent, +, present.

The DNA present in plasmid pSE1004 but not in plasmid pSE1009 was subcloned into plasmid vector pTrc99A to yield plasmid pAG1. The introduction of this plasmid into strain SE1811 resulted in full restoration of the molybdate-dependent repressor activity as shown by the low level of *modA - lacZ* expression for strain SE1811(pAG1) with the addition of 1 mM sodium molybdate to the growth medium. The lower level of *modA - lacZ* expression for SE1811(pAG1) in the absence of added molybdate, (600 units of β-galactosidase activity as opposed to 1 500 units for strain SE1811), is most likely due to the presence of multiple copies of the plasmid in each cell. Presumably when there is this higher dosage of repressor in strain SE1811 cells as a result of the presence of plasmid pAG1 any trace amount of molybdate present in the cell would serve to enhance the partial repression of the *modA - lacZ* seen under these conditions.

Based on the results presented in Tables 3 and 4 it can be concluded that there is a molybdate-dependent repressor which acts to limit expression of the *modABCD* operon when a sufficient intracellular concentration of molybdate is sensed. The gene that presumably codes for this repressor is designated *modE* and the *modE* gene is located adjacent to the *modABCD* operon but is transcribed in the opposite direction compared to the *modABCD* operon.

Analysis of *modEF* DNA

modE gene. The region of DNA which complemented the mutation in strain SE1811 was sequenced and a two-gene operon starting 444 bp upstream of the *modA* translation start site was identified (Fig. 2 modified from 60). These two open reading

Fig. 2. DNA sequence of the *modEF* operon. Amino acid sequences are listed below the DNA sequence. Presumptive −10 and −35 sequences are underlined and ribosomal binding sites are double underlined. Asterisks denote translation terminations. The location of the *lacZ* insertion in the *modE* gene in plasmid pRM28 is indicated by the down arrowhead. The stem-loop structure is shown by the two opposing arrows. ATP-GTP binding motifs in *ModF* are in boldface type.

frames, designated *modE* and *modF* are transcribed in the opposite direction as compared to *modA* and encode a 262-amino-acid protein (28 200 Da) and a 490-amino-acid protein (53 900 Da) respectively An eight-bp-stem six-bp-loop can be identified in the DNA between the operator/promoter regions of *modA* and *modE* and is indicated by the two opposing arrows in Fig. 2 This DNA is located between *gnl* and *modABCD* operons in the *E coli* chromosome and the *galETK* operon is transcribed in the same direction as the *modEF* operon.

A search for proteins that bear similarity to ModE was conducted resulting in the detection of four homologs (Fig. 3) The homologs were identified as ModA from *A vinelandii* (35% identity 45% similarity) (43) (renamed ModE [50]), ModE from *H influenzae* Rd (48 % identity, 58% similarity) (20), MopA from *R. Capsulatus* (34% identity 46% similarity) (76), and MopII from *C pasteurianum* (29% identity 46% similarity) (32) A possible helix-turn-helix motif which is a known prokaryotic DNA-binding protein structure motif (52) has been identified in the N-terminal part of the *E. coli* ModE as indicated by the underlined sequence in Fig. 3

Since *H influenzae* ModE is almost 60% similar to *E coli* ModE and both are facultative anaerobes, it was thought that the *H influenzae* ModE homolog may be able to substitute for the *E. coli* ModE protein in the ModE deficient *E. coli* strain SE1811 This possibility was tested by monitoring the *modA – lacZ* expression in strain SE1811 which had been transformed with a plasmid containing the *H influenzae modE* gene

Fig. 3 Amino acid sequences of the homologs of *E. coli* ModE protein. AvModE, *A vinelandii* ModE (43) EcModE *E. coli* ModE HiModE, *H influenzae* Rd (20) KcMopA, *K. capsulatus* ModE (76) CpMopII *C pasteurianum* MopII (31) Double dots represent identity, and single dots represent conservative substitutions. Shadow print indicates the conserved SARNQ sequence. All identity and similarity designations are in relation to *E. coli* ModE A presumptive helix turn-helix DNA binding region in *E coli* ModE is underlined

(plasmid plsgG) A low level of *modA – lacZ* expression was seen for strain SE1811(plsgG) that was cultured in LBG supplemented with 1 mM sodium molybdate (Table 5) verifying that the *H. influenzae* ModE protein can functionally substitute for *E. coli* ModE.

modF gene. Expression of the *modF* gene from a T7-based expression vector plasmid pRM23 yielded a 54 kDa protein when visualized after SDS-PAGE. This size is in agreement with the expected size based on the DNA-sequence derived amino acid sequence. Database searches revealed no homologs of *E. coli* ModF protein, but further examination of the amino acid sequence identified two ATP-GTP binding site motifs (Fig 2).

As of yet, the function of *modF* has not been determined, since deletions of the *modF* gene did not result in a detectable phenotype for FHL production or *modA* gene expression or activity under the conditions used in this study. A study by Dorrel et al. (17) has described and presented the sequence of a gene involved in photoreactivation (*phrA*). This DNA sequence is situated within the *modF* gene presented in Fig. 2. Based on their studies, Dorrel et al. proposed that the 38 kDa PhrA protein is necessary for photorepair. However, the findings that a 54 kDa protein and not a 38 kDa product was observed when *modF* was expressed in a T7 RNA polymerase-driven expression system and that the expression of ϕ(*modF'-'lacZ*) responds to regulation by ModE (data presented later) suggest that the DNA in question codes for *modF*.

Table 5 Complementation of the *modE* mutation in strain SE1811 with *H. influenzae modE*

Strain	Relevant genotype	β-galactosidase Activity[a] -Mo	+Mo
SE1811	ϕ(*modA lacZ*), *modE*	1,300	1,300
SE1811(pIogG)	ϕ(*modA - lacZ*), *modE*, (H.i. *modE*$^+$)	1 500	< 10

[a]Cells were grown in LBG medium. Sodium molybdate concentration was 1 mM. β-galactosidase activities are expressed as nanomoles minute milligram of cell protein

Regulation of *modA* by ModE

In order for the interaction of ModE with its target operon *modABCD* to be investigated, the ModE protein first had to be purified. ModE protein was overexpressed and purified as indicated in "Materials and Methods" section. The protein yield after each purification step is shown in Table 6 while the purity of ModE after each purification procedure is presented in Figure 4. A final yield of purified ModE protein of 5% of total protein in the extract was obtained, and this protein was judged to be pure by native polyacrylamide electrophoresis (not shown) SDS-PAGE (Fig. 4) and N-terminal amino acid sequencing of the protein. The first twelve amino acids are MQAEILLTLKLQ and these correspond to the predicted amino acid sequence of the protein (Fig. 3). The molecular mass of the purified protein was determined to be 28 271 Da using matrix assisted laser desorption ionization time-of-flight mass spectrophotometry. This mass value is in agreement with the predicted value of 28,200 Da based on the DNA sequence-derived amino acid sequence as well as a value of 29,000 Da obtained after SDS-PAGE.

Coupled in vitro transcription-translation experiments. Experiments in which the β-galactosidase activity of *modA::lacZ* was monitored in two isogenic strains, one having a functional *modE* gene (strain SE2069) and the other carrying an inactivating mutation in *modE* (strain SE1811) suggested that ModE represses transcription of *modA::lacZ* and presumably the *modABCD* operon in a molybdate-dependent fashion (Table 4). A direct approach to investigating the interaction of ModE with its target DNA is to determine the

Table 6 Purification profile of ModE

Fraction	Total protein (mg)	Purification (fold)
Extract	238	1 0
Q Sepharose fast flow	57	4 2
HiTrap Heparin	16.5	14 4
Chromatofocusing	12	19 8

Fig 4 SDS PAGE analysis of proteins from different stages of MedE purification Lane 1 molecular weight markers (from top phosphorylase b 97 400 bovine serum albumin, 66 200 ovalbumin, 45 000, carbonic anhydrase 31 000 trypsin inhibitor 21 500 lysozyme, 14,400), lane 2, extract lane 3 proteins after Q Sepharose fast-flow purification lane 4 proteins after HiTrap Heparin purification, lane 5, proteins after chromatofocusing A 25-μg amount of protein was loaded into each of lanes 2-4 A 10-μg amount of protein was loaded into lane 5

level of expression of target genes (*modAB'*) with and without the effector (ModE) in an in vitro coupled transcription-translation experiment with S30 extract.

For these coupled transcription-translation experiments, plasmid pRM1 DNA, containing *modAB'* was incubated with *E. coli* S30 extract with and without 1 mM sodium molybdate and 40 pmol of ModE. In these experiments, the molybdate concentration was maintained at 1 mM since the affinity between the protein and molybdate is not known. Also, it is not known whether the Mo species bound to ModE is molybdate or some other derivative of molybdate. It should be noted that the S30 extract provides all the constituents necessary for production of proteins from a DNA template and that addition of ^{35}S-methionine allows for labeling of proteins produced in the reaction mixture. The resultant autoradiogram (Fig. 5) and derivative Phosphorimager data indicate that the presence of ModE in the reaction mixture reduced production of the 26-kDa ModA protein and the 24-kDa ModB protein by 48 and 65%, respectively (lanes 1 and 2). In Fig. 5, two bands are labeled as ModA. ModA, which has been shown to function as a molybdate-binding protein (59), has a putative 24 amino acid leader signal peptide thought to be responsible for its localization in the cell's periplasmic space (60). Given that ModA has a signal peptide, the upper band would be the unprocessed modA while the lower band would be ModA without its leader peptide. With the addition of sodium molybdate, repression of *modAB'* expression was even greater with over 90% decrease in production of ModA and ModB' (Fig. 5, lanes 3 and 4). The decrease in production of ModA and ModB' even in the absence of added sodium molybdate is most likely due to the presence of contaminating molybdate in the S30 extract. It should also

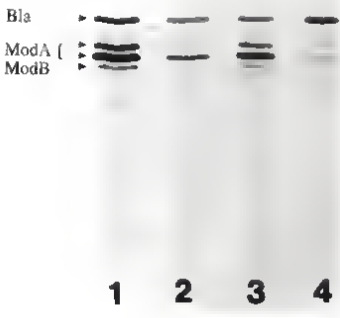


Fig 5 SDS-PAGE analysis of proteins produced in an in vitro transcription-translation experiment in which plasmid pRM1 served as the template DNA Sodium molybdate and ModE concentrations were 1 mM and 40 pmol per reaction mixture, respectively Lane 1 without ModE, lane 2 with ModE lane 3 without ModE, with sodium molybdate lane 4, with ModE and sodium molybdate, Bla, β lactamase The upper ModA protein band represents the precursor protein bearing the signal peptide while the lower ModA protein band represents the protein with the signal peptide removed ModB truncated ModB protein.

be pointed out that, although equal amounts of radioactivity were loaded in each lane, the samples in lanes 2 and 4 appear to contain a total of less labeled protein than is present in the other lanes. This disparity in the amounts of labeled protein is due to the presence in these samples of low molecular weight (partially degraded?) protein products which migrate faster than the dye front during electrophoresis. Addition of molybdate alone did not significantly reduce the amount of ModA and ModB produced in the reaction mixture. Thus, the reduction in the production of ModA and ModB proteins with ModE in the reaction mixtures confirms that ModE acts as a repressor of the *modABCD* operon and that this repression is enhanced by molybdate.

In an effort to further show that ModE was binding to the *modA* operator/promoter DNA and thereby reducing expression of the operon, a 42-bp DNA fragment, believed to encompass the ModE target binding region, was added to the transcription-translation reactions along with the ModE protein in order to titrate out ModE and relieve the repression of *modAB*. The added DNA is a 42-bp DNA derived from the operator/promoter region of *modA* spanning -9 to +33. With the addition of 50-fold more 42-bp oligomer as compared to plasmid pRM1 in the reaction mixtures, production of ModA was restored to 66% of the levels seen without ModE, and inclusion of 100-fold excess of 42-bp oligonucleotide fully restored production of ModA and ModB to the levels seen in the absence of ModE (data not shown). Clearly, even though the DNA used to compete for the binding of ModE carried only a part of the operator/promoter region of the *modABCD* operon, the 50-fold and 100-fold excess of this DNA compared to the

template DNA present in the reactions was sufficient to eliminate efficient binding of ModE to plasmid-borne target DNA.

Characterization of the Interaction of ModE with *modA* Operator/promoter DNA

Determination of ModE's target binding site sequences using in vivo titration experiments. The rationale for this series of experiments aimed at identifying the region of DNA in the *modA* operator/promoter region required for successful binding of ModE protein is based on the idea that the presence of a large number of copies of DNA, in the form of plasmid DNA, containing the repressor's binding site(s) should result in the binding of a certain fraction of the repressor molecules produced from the chromosomal copy of *modE*. With the repressor molecules bound to the plasmid DNA, and thus not available as free protein, repressor binding at its target site on the chromosome should be reduced. This reduction of binding to the chromosomal ModE binding site can be monitored using an appropriate *modA - lacZ* fusion in the chromosome. More specifically, various lengths of DNA ranging from -247 to +25 of the *modA* operator/promoter region were cloned into the multiple cloning site of the high copy plasmid vector pUC19, as described in "Materials and Methods" section. The resultant plasmids were subsequently transformed into strain SE2069 which contains a chromosomal ϕ(*modA - lacZ*).

An initial inspection of the *modA* operator/promoter DNA suggested that perhaps an eight-bp inverted repeat (TAACGTTA) spanning the +4 to +11 region might be involved since inverted repeats had been shown to function as the binding sites for the *trp* and *met* repressors (40, 54, 55). However, a closer inspection of the *modA* operator/

promotin DNA revealed that there is a preponderance of GTTA sequences in this DNA (Fig. 6) Specifically, there are 8 GTTA tetramers between the translation start sites of *modA* and *modE* and all are located near the *modA* end It is especially notable that two of the GTTA tetramers straddle the -10 region, and that there is a GTTA tetramer inverted repeat (the eight-bp inverted repeat referred to above) located 4-bp downstream of the transcription start site Thus the number and location of these GTTA tetramers provided compelling reasons for investigating their potential role in the binding of ModE

For determination of the degree of titration of ModE by the various plasmids, β-galactosidase assays were performed as per standard protocol, and ampicillin was added to the culture medium to ensure the maintenance of the plasmids in the cells Supplementation of 1 mM molybdate to the culture medium was also required since strain SE2069 cells are functionally Mod , thus, in the absence of added molybdate ModE fails to serve as a repressor of *modA - lacZ* expression (Table 3) The resulting β-galactosidase activities are presented in Table 7 The *mod* DNA sequence that is present in each of the plasmids is also indicated in Table 7 It should be noted that the data is presented in a manner between plasmids pAM7 to pAM5 that the first β-galactosidase activity corresponds to the construct bearing all of the GTTA tetramers (plasmid pAM7), while each following plasmid has successive GTTA sequences removed from left to right respectively until plasmid pAM5 is reached

The level of *modA lacZ* expression for strain SE2069 with plasmid pAM4, which contains the entire intergenic DNA sequence shown in Fig. 6, is 510 β-galactosidase activity units while expression for SE2069 cells alone or for SE2069 harboring plasmid

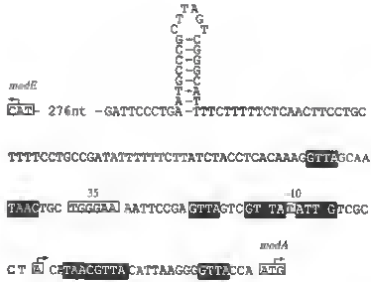


Fig. 6 Intergenic region of *modEA* DNA. Boxes outline the *modA* -10 and -35 sequences. The *modA* transcription start site is indicated by the boxed A with the arrow and the translation start site appears as the boxed ATG or 'CAT' 'GTTA' sequences are shown in reverse print

Table 7 Titration of ModE protein in vivo by various DNA sequences in strain SE2069 (*modA*$^{-}$ *lacZ*$^{+}$)

Plasmid	DNA Sequence	β-Galactosidase Activity
No Plasmid		150
pAM4	GGTACC- 186nt - CAAAGTTACAATAAGTGCTGGGAAAATTCC- GATTTATGTCGTTATTCGCCTACATAACGTTACATTAAGGGTTAC 3 6 5 6 7 8	510
pAM5	GGTACC- 186nt -	140
pAM7	CAAAGTTACAATAAGTGCTGGGAAAATTCC- GATTTATGTCGTTATTCGCCTACATAACGTTACATTAAGGGTTAC	510
pAM8	AAATTCC GATTTATGTCGTTATTCGCCTACATAACGTTACATTAAGGGTTAC	710
pAM13	TTTATTCGCCTACATAACGTTACATTAAGGGTTAC	750
pAM10	TATTCGCCTACATAACGTTACATTAAGGGTTAC	150
pAM6	TCGCCTACATAACGTTACATTAAGGGTTAC	120
pAM5	CGCCTACATAACGTTACATTAAGGGTTAC	60
pAM15	GGTACC 186nt - CAAAGTTACAATAAGTGCTGGGAAAATTCC- GATTTATGTCGTTATTCGCCTACA ------CATTAAGGGTTAC	80
pAM16	GGTACC 186nt - CAAAGTTACAATAAGTGCTGGGAAAATTCC GATTTATGTCGTTATTCGCCTACATAACGTTACATTAAGGG---C	410

Cells were grown in LBG medium with molybdate (1 mM) β-galactosidase activities are expressed as nanomoles-minute^{-1}-milligram of cell protein^{-1} The putative ModE protein binding sites are indicated by inverse print and are numbered (pAM4)

pAM9 which contains only the 5' 192 bp of the intergenic region is 150 and 140 units, respectively. These data indicate that the *modEA* intergenic region is capable of titrating a fraction of ModE thereby relieving repression of the chromosomal *modA - lacZ* and that the first 192 bp of the insert in plasmid pAM4 is not necessary for this titration. Based on the results presented in Table 4 a β galactosidase activity of 1,300 units is expected for strain SE2069 under conditions of derepression, thus, the relief of repression of *modA - lacZ* by plasmid pAM4 seen in the titration experiments is not total, but is of sufficient level to discriminate the sequence required for the binding of ModE. Removal of the first two GTTA tetramers resulted in an increased titration of ModE by the plasmid DNA as shown by the increase in β-galactosidase activity from 510 units to 710 units when plasmid pAM8 is present rather than plasmid pAM4 or pAM7. Likewise removal of the next GTTA tetramer (plasmid pAM13) yields no even higher level of titration, 750 β-galactosidase units as opposed to 710. The higher β-galactosidase activities obtained using plasmids pAM8 and pAM13 suggest that the first three GTTA tetramers may interact with ModE forming unstable associations, which results in an overall less efficient binding of ModE to its true target binding regions. The fact that these first three GTTA tetramers do naturally exist in the *modEA* intergenic region implies that perhaps these GTTA tetramers help to modulate the repression of the *modABCD* operon by ModE under suboptimal intracellular molybdate concentrations.

Removal of the first four as well as the first four and part of the fifth GTTA tetramers (plasmids pAM10 and pAM6, respectively) results in the complete loss of titration of ModE, which demonstrates that the fourth and fifth GTTA tetramers are

necessary for successful binding of ModE. Moreover, these two GTTA tetramers are the ones that are situated in the *modA* -10 sequence, and would be expected to participate in binding of the repressor given that binding of a repressor at that point would serve to obstruct the binding of RNA polymerase and thereby limit transcription of the ensuing gene. Furthermore, removal of only the sixth and seventh GTTA tetramers (pAM15) also results in complete loss of titration as indicated by the drop in β-galactosidase activity to 90 units. This finding establishes the requirement for the sixth and seventh GTTA tetramers for ModE binding, and again the location of these two GTTA sequences 4-5p downstream of the transcription start site, supports the assertion that these sites are integral to the binding of repressor. Additionally, the fact that the binding of ModE to the DNA requires the presence of the fourth and fifth as well as the sixth and seventh GTTA tetramers intimates that the binding of ModE to one set of sites necessitates the initial binding of ModE to the other site. This potential binding order in turn evokes the possibility of cooperative binding occurring; however, none of the methods used in this study to characterize the binding of ModE to its target DNA is capable of evaluating cooperativity. Introduction of plasmid pAM16, which only lacks the eighth GTTA sequence, into strain SE2069 results in a partial loss of titration of ModE, thereby implicating the involvement of this eighth tetramer in stabilizing ModE once it is bound to the DNA or possibly aiding in the initial recruitment of ModE.

Based on these in vivo titration experiments, it has been determined that the binding of ModE minimally requires the presence of the fourth, fifth, sixth, and seventh GTTA tetramers, while the presence of the eighth GTTA tetramer increases the efficiency

of binding. Furthermore the arrangement of these GTTA tetramers required for binding (the fourth and fifth GTTA tetramers [GTTATATTG] seem to constitute one binding site, while the sixth and seventh GTTA tetramers [TAACGTTA] could form a second site) and the size of ModE protein (28,271 Da) predicts that ModE likely binds at each of the two sites as a dimer. The differences in the sequences also suggests different binding efficiencies.

In vitro studies characterizing the binding of ModE to its target DNA. Having established putative binding sites for ModE using the in vivo titration experiments, in vitro techniques such as DNA-mobility shift assays and DNase I-footprinting experiments were undertaken in an effort to confirm the in vivo findings as well as to provide further information on the kinetics of the interaction. One set of experiments designed specifically to determine whether the in vivo titration results would be mirrored by DNA mobility shift assay results, involved preparing binding reactions containing various amounts of ModE and ^{32}P-labeled DNA-oligomers derived from the *mod* DNA in plasmids pAM13, pAM15 and pAM16. In these experiments, a 211 bp *DraI*/*Eco*RI fragment derived from plasmid pAM13 (40 bp of *modA*-operator/promoter DNA from -15 to +25 and 171 bp of plasmid pUC19) and a 313 bp *Eco*RI/*Hind*III fragment from plasmid pAM15 (272 bp of *modA* operator/promoter DNA from -247 to +25 and 41 bp plasmid pUC19 DNA) and a 305-bp-*Eco*RI/*Hind*III fragment from plasmid pAM16 (267 bp *modA*-operator/promoter DNA from -247 to +20 and 38 bp plasmid pUC19 DNA) was used as the source of *mod* operator/promoter DNA. Standard DNA mobility shift assay protocol outlined in "Materials and Methods" section was followed, and the results are presented in Fig. 7

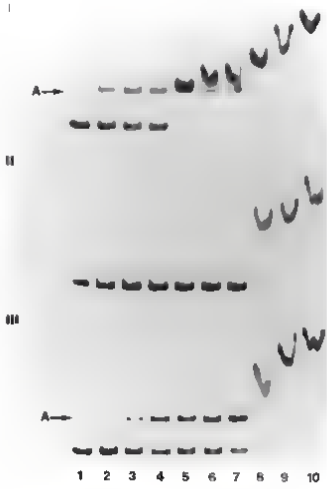


Fig. 7. DNA mobility shifts using plasmid pAM13-derived (panel I), plasmid pAM15-derived (panel II) and plasmid pAM16-derived DNA (panel III) and ModE. A 0.1 pmol amount of labeled DNA and 1 mM sodium molybdate were present in each binding reaction. The following amounts of ModE had been added to the reactions as indicated. lane 1, without ModE; lane 2, 1 fold excess ModE compared to DNA; lane 3, 5-fold excess; lane 4, 10-fold excess; lane 5, 25-fold excess; lane 6, 50-fold excess; lane 7, 100-fold excess; lane 8, 250-fold excess; lane 9, 500-fold excess; lane 10, 1000-fold excess. The arrow indicates a "biologically significant" shifted band.

In the DNA mobility shift for the plasmid pAM13-derived DNA (Fig 7 panel I) a detectable shift to position 'A' (complex A) occurred at a protein to DNA ratio of 1 0 (25 7% DNA shifted to form 'A) This shift form predominated until a ModE excess of 25-fold was reached, at which point, a second band begins to emerge Other ModE-DNA complexes also emerged at 250-fold 500-fold, and 1000-fold ModE excess These different complexes most likely represent additional binding of ModE to the DNA in a nonspecific manner with little biological significance When DNA is included in the binding reactions which is incapable of titrating ModE (plasmid pAM15-derived DNA Table 7), shift-form A ModE-DNA complex does not arise (Fig 7, panel II) but the higher-mass shift complexes did arise at ModE excesses of 250 500 and 1000-fold Thus, the DNA-mobility results, combined with the inability of plasmid pAM15 to titrate ModE in the in vivo experiments (Table 7) indicates that the 'A complex form is the DNA-target-specific form. Based on the ModE titration data (Table 7) plasmid pAM16-derived DNA should prove capable of forming the shift form A ModE-DNA complex, albeit at higher ModE/DNA ratios than required for plasmid pAM13-derived DNA-ModE complex formation. This expectation was realized in the DNA mobility shift experiments, as complex A did form but was only first detectable at a ModE to DNA ratio of 5 0 and over 50% shift of DNA did not occur until 100-fold excess ModE to DNA (Fig 7 panel III) Again, at higher ModE concentrations, 250 500 and 1000-fold excesses, the larger ModE DNA complexes arose with this DNA also

In all three of the DNA mobility shifts presented in Fig. 7 multiple ModE DNA complexes other than the complex identified as shift-form 'A' are observed Since these

ModE DNA complexes arise regardless of whether the DNA is capable of titrating ModE it is thought that these complexes result from non-specific binding of ModE to non-*mod* sequence or to *mod* sequence located upstream of the *modA*-operator/promoter when there is greater than 250 fold excess ModE to DNA in the binding reaction. In order to determine whether the non-*mod* DNA contributes to formation non-specific ModE DNA associations, a DNA-mobility shift experiment was conducted in which a 42-bp oligomer (*modA*-operator/promoter DNA from -9 to +33) that is essentially the sequence present as the insert in plasmid pAM10 (*modA*-operator/promoter DNA from -10 to +25) was used. As indicated in Table 7 this particular DNA does not titrate ModE and, therefore, should not support formation of complex 'A'. Results of this DNA mobility shift experiment are presented in Fig. 8 and as can be seen, DNA was not shifted at any ModE concentration used except at 1000-fold excess ModE compared to 42-bp oligomer. Furthermore, this shifted DNA protein complex migrated at a much slower rate than the shift-form 'A' observed with DNA from plasmid pAM13 (Fig. 7 panel I). Inclusion of a smaller DNA fragment (42 bp, as opposed to 211 or 305 or 313 bp), which does not contain non-*mod* DNA, eliminated formation of the larger ModE-DNA complexes at all ModE concentrations except at the very high 1000-fold excess ModE. This result supports the assertion that the interaction of ModE with non-*mod* DNA at high ratios of ModE to DNA leads to the formation of non-specific ModE-DNA associations.

The DNA mobility shift experiments (Figs. 7 and 8) have demonstrated that the formation of the ModE-DNA complex (complex 'A') in vitro requires the presence of the fourth, fifth, sixth, and seventh GTTA tetramers, and that the eighth tetramer is necessary

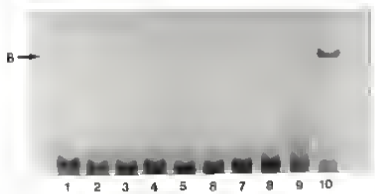


Fig 8 DNA-mobility shift featuring 42-bp *modA* operator/promoter DNA (9 to +33) and ModE A 0 1 pmol amount of DNA and 1 mM sodium molybdate and the following amounts of ModE were included in each binding reaction. Lane 1 without ModE lane 2, 1-fold excess ModE compared to DNA lane 3 5-fold excess, lane 4 10 fold excess lane 5 25 fold excess, lane 6 50 fold excess; lane 7 100-fold excess lane 8 250-fold excess lane 9 500-fold excess, lane 10 1000 fold excess.

for efficient binding (Table 7, Fig. 7). The mobility shift experiments fully support the results from the in vivo ModE titration experiments (Table 7), further lending credence to the use of the titration experiments as a means of probing the association of protein with its target DNA sequence.

It has already been shown both in vivo and in vitro that repression of the *modABCD* operon by ModE requires the presence of intracellular molybdate (Tables 3 and 4, Fig. 5). Therefore, DNA mobility shift experiments were conducted for the purpose of establishing whether molybdate is required for the binding of ModE to its target DNA. For these DNA-mobility shift experiments, a 43 bp oligonucleotide (−18 to +25), was used in the binding reaction along with the appropriate amount of ModE protein. For one set of shifts, sodium molybdate was added to the binding reactions, to the gel the binding reaction samples were electrophoresed in, and to the electrophoresis tank buffer at a final concentration of 1 mM. For the second set of mobility shifts, molybdate was excluded. These two mobility shifts, presented in Fig. 9, indicate that molybdate is necessary for efficient binding of ModE, as the 'A' complex is first detectable at a ModE/DNA ratio of 1.0 in the presence of 1 mM sodium molybdate, whereas in the absence of molybdate, the ModE-DNA complex was not detectable until a 100-fold excess ModE was included in the binding reaction. Furthermore, binding of 50% or greater of DNA with ModE was never achieved in the binding reactions lacking molybdate, while in the presence of 1 mM sodium molybdate, greater than 50% shift of the DNA to the ModE-DNA complex 'A' was obtained at a ModE/DNA ratio of 50

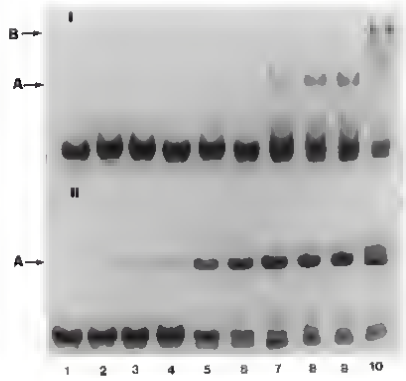


Fig 9 DNA mobility shifts using a 43-bp oligomer spanning the −17 to +25 region of the *modA*-operator/promotor region and ModE Panel I=sodium molybdate was not added to the binding reaction, gel or running buffer Panel II= 1 mM sodium molybdate was present in the binding reaction, gel, and running buffer A 0 1 pmol amount of DNA was present in the binding reaction. The following amounts of ModE were present in the binding reaction samples as indicated Lane 1 without ModE lane 2, 1 fold excess ModE compared to DNA lane 3 5 fold excess, lane 4, 10 fold excess, lane 5 25 fold excess lane 6, 50-fold excess lane 7 100 fold excess, lane 8 250-fold excess, lane 9 500-fold excess, lane 10 1000-fold excess

The apparent dissociation constant (K_D) for the binding of ModE to the 43-bp oligomer in the presence of 1mM molybdate was determined after quantitation of the percent of shifted DNA using a Phosphorimager. This correlation is presented in Fig. 10 and an apparent K_D of 0.33 μM was calculated for the ModE-DNA complex "A". An apparent K_D for the binding of ModE to the 43-bp DNA in the absence of 1 mM sodium molybdate could not be calculated since even at 1,000 fold excess of protein, the amount of shifted DNA was less than 50% of the total DNA.

Interaction of molybdate with ModE. Given that the presence of molybdate is required for efficient binding of ModE to its target DNA, the concentration of molybdate needed for a 50% shift of the 43 bp oligomer to the form "A" ModE-DNA complex was determined. A ModE concentration of 0.5 μM (protein to DNA ratio of 50) was chosen for these shift experiments because at this ModE concentration in the binding reaction, a 60% shift of the DNA was obtained in the presence 1 mM sodium molybdate, and at this ratio, the contribution of sodium molybdate to the ModE-DNA interaction would be readily discernable.

Based on these experiments, a 50% shift of the 43 bp oligomer was accomplished at a molybdate concentration of 6 μM (Fig. 11). It is not unreasonable to consider that an artificially high apparent K_D value for the association of molybdate with ModE could have been obtained given the inherent limitations of the experiment used to derive the apparent K_D. Specifically, for the molybdate concentration DNA mobility shift experiments, once the binding reaction samples were loaded onto the gel, the effective concentration of sodium molybdate present in these samples would decrease as the sodium molybdate

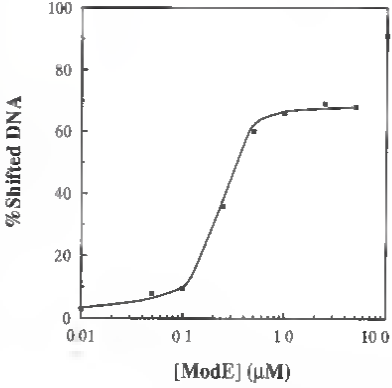


Fig 10 Percentage of ModE-43-bp-oligomer complex 'A' formed versus concentration of ModE protein in the presence of 1 mM sodium molybdate These values are from the DNA mobility shift experiment presented in Fig 8 panel II

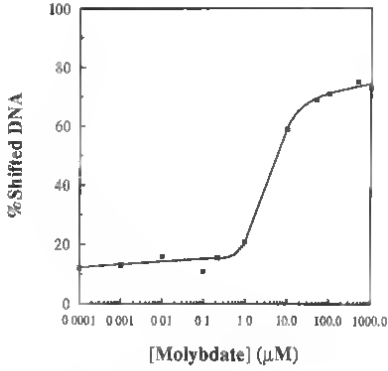


Fig 11 Percentage of 43-bp-oligomer bound to ModE protein in relation to the concentration of sodium molybdate present in the binding reactions in a DNA-mobility shift experiment

concentration for the entire system undergoes equilibration, and secondly electrophoresis of the binding reaction complexes may perturb the ModE-molybdate association

If the actual binding of ModE to its target DNA constitutes the rate-limiting step of the association of ModE with the DNA rather than the interaction of molybdate with ModE then this concentration of molybdate (6 μM) required for half-maximal binding most likely represents an upper limit value since it has previously been shown that the apparent dissociation constant for the interaction between ModE-molybdate and *mod*-operator/promoter DNA is 0 3 μM

If however it is the association of molybdate with ModE and not the binding of ModE to the DNA which serves as the rate limiting step, then the 6 μM apparent K_D value for the dissociation of molybdate from ModE may indeed prove valid. This possibility is especially compelling in light of the K_D value of 3 μM that was determined for the affinity of molybdate for the periplasmic molybdate-specific binding protein, ModA, using fluorescence studies (59) Furthermore an apparent K_D of 6 μM for the binding of molybdate to ModE seems physiologically acceptable since molybdate entering the *E coli* cell would preferentially be activated and ultimately incorporated into an appropriate apo-molybdoenzyme. Under these physiological conditions, the apparent K_D value for molybdate in ModE-system would be expected to be higher than the K_M value for molybdate for the activation enzyme. Only when the internal molybdate saturates the biosynthetic pathway, would molybdate be available for association with ModE resulting in the repression of the *modABCD* operon and cessation of further production of the components of the molybdate-specific transporter If the apparent K_D for the dissociation

of molybdate from ModE were much lower, then it would be likely that molybdate's interaction with ModE would be favored above its activation and incorporation into molybdoenzymes which would prove physiologically untenable for the cell. Given the uncertainty of whether it is the interaction of molybdate with ModE or it is the binding of activated ModE to DNA which acts as the limiting step for the repression, it seems that the only way to establish an accurate description of the kinetics governing the association of molybdate with ModE and in turn, the binding of the ModE-molybdate complex to its target DNA is to determine the interaction in solution and compare to the appropriate enzymes competing with ModE for molybdate.

Oxyanion specificity of ModE. Having established that molybdate is required for the repression of the *modABCD* operon in vivo and the efficient binding of ModE to its target DNA in vitro, the question of whether structural analogs of molybdate could substitute for molybdate was addressed. This question was answered in two ways: (1) *modA – lacZ* expression in strain SE2069 cells that were cultured in LBG medium supplemented with 1 mM sodium molybdate, 1 mM sodium tungstate, 1 mM sodium sulfate, or 1 mM sodium ortho-vanadate; and (2) DNA-mobility shift experiments in which the binding reactions, gels, and tank buffers contained 10 μM sodium molybdate, 10 μM sodium tungstate, 10 μM sodium sulfate, or 10 μM sodium ortho-vanadate.

The resulting levels of *modA – lacZ* expression in strain SE2069 cultured with the various oxyanions are presented in Table 8. Only in those cultures of strain SE2069 grown in medium supplemented with 1 mM sodium molybdate or 1 mM sodium tungstate did repression of the *modABCD* operon occur, as indicated by their low β-galactosidase

Table 8 Expression of *modA – lacZ* in strain SE2069 cultured in the presence of various oxyanions.

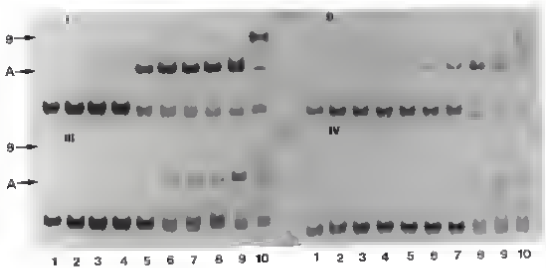
Strain	Relevant Genotype	Oxyanion Added	β-galactosidase Activity[a]
SE2069	ϕ(*modA – lacZ*)	None	1,300
		MoO_4^{-2}	30
		WO_4^{-2}	40
		SO_4^{-2}	1,400
		VO_4^{-3}	1 900

[a]Cells were grown in LBG with the appropriate oxyanion at a final concentration of 1 mM. Enzyme activities are expressed in nanomoles minute^{-1} milligram of cell protein^{-1}

activities. Therefore it appears that tungstate is the only oxyanion tested which is capable of functionally substituting for molybdate in the molybdate-dependent activation of ModE in vivo. Furthermore, the fact that higher *modA—lacZ* expression levels were observed when the cells were grown in LBG medium containing sodium sulfate and especially sodium vanadate compared to cells grown in medium without any oxyanion supplementation (1 400 and 1 900 β-galactosidase units, respectively, as opposed to 1,300 units) implies that perhaps the presence of these two oxyanions inhibits the interaction of ModE with any of the trace amount of molybdate that may have been present in the cells, thereby reducing binding of ModE-molybdate complex to the *modA*-operator/promoter region.

The results of the DNA-mobility shift experiments (Fig. 12) which contained 10 μM amounts of the previously indicated oxyanions in the binding reactions, were consistent with the in vivo *modA—lacZ* expression data (Table 8). More specifically, high affinity binding of ModE to the 43 bp DNA and shift to complex 'A' only occurred in the presence of sodium molybdate (Fig. 12, panel I) or sodium tungstate (Fig. 12, panel II) while a low level of binding was seen for the shifts with sodium sulfate (Fig. 12, panel III). Sodium vanadate was fifty times less effective in supporting DNA-ModE protein interaction (Fig. 12, panel IV). The apparent K_D values for the ModE-DNA complex obtained from the correlation of percent of shifted DNA (ModE-DNA complex species 'A') versus the concentration of ModE for the shifts with the different oxyanions were as follows: 0.22 μM with 10 μM sodium molybdate supplementation, 1.3 μM with sodium tungstate, and 4.7 μM with sodium sulfate (Fig. 13). An apparent K_D value for the

Fig. 12. DNA mobility shift experiment in which four different oxyanions were present in the binding reactions. The binding reactions contained 10 μM sodium molybdate (panel I), 10 μM sodium tungstate (panel II), 10 μM sodium sulfate (panel III), or 10 μM sodium ortho vanadate (panel IV) along with 0.1 pmol of the 43-bp *modA* operator/promoter DNA. The following concentrations of ModE were present in the binding reactions per lane as indicated: lane 1, no ModE; lane 2, 1 fold excess ModE compared to DNA; lane 3, 5-fold excess; lane 4, 10-fold excess; lane 5, 25 fold excess; lane 6, 50-fold excess; lane 7, 100-fold excess; lane 8, 250-fold excess; lane 9, 500-fold excess; lane 10, 1000 fold excess.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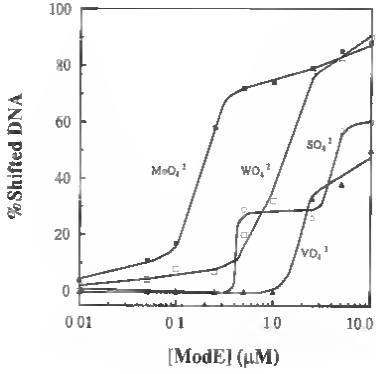

Fig. 13 Percent shifted DNA versus concentration of ModE resulting from DNA mobility shift binding reactions containing various oxyanions. Binding reactions included 10 μM sodium molybdate (filled squares) 10 μM sodium tungstate (open squares), 10 μM sodium sulfate (open circles) or 10 μM sodium ortho-vanadate (closed triangles).

binding of ModE in the presence of 10 μM sodium vanadate could not be calculated since 50% shift of the DNA was not achieved with the ModE concentrations used. It is not surprising that tungstate proved to be the only oxyanion of those tested which was capable of substituting for molybdate in activating ModE to any significant degree since it has previously been shown that activated tungstate can be incorporated successfully into some *E. coli* apo-molybdoenzymes in place of molybdate (57). However these tungstate-substituted molybdoenzymes are not functional probably because of a failure of electron transfer across the tungsten center. Yet, the stable association of tungstate with ModE would result in the promotion of the activation of ModE since presumably, the activation of ModE relies only on the binding of an appropriately sized and shaped moiety to affect the change in conformation of the protein which allows for the efficient binding of DNA, and does not require any further chemical process such as the transfer of electrons.

Stoichiometry of ModE-DNA complex. Since a stable ModE-43-bp DNA shift complex (complex form 'A') has been reproducibly obtained under a variety of conditions, the general method was used to determine the molecular weight of the ModE DNA complex which would then indicate the number of ModE proteins which are associated with the DNA in complex 'A'. For the molecular weight determination of the ModE-DNA complex, standard binding reaction samples supplemented with 1 mM sodium molybdate were electrophoresed in 5%, 6%, 7%, and 8% nondenaturing-polyacrylamide TBE gels. The resulting R_f values for ModE-DNA complex and the protein standards after migration through the four different gels were used to prepare a Ferguson plot (19) from which the molecular weight of the ModE-DNA complex could be

extrapolated (Fig. 14). Based on the results of these experiments, a value of 81,247 Da was obtained for the size of this complex. This molecular weight compares favorably with a molecular weight of 83,144 expected for the association of a ModE-dimer (56,424 Da) with the 43-bp DNA (26,602 Da), as there is only a 2.3% difference in the expected value versus the observed molecular weight. Thus, the ModE-DNA complex formed is minimally a ModE dimer bound to the 43-bp *modA*-operator/promoter DNA. In these experiments, the axial ratio and electrophoretic mobility of the protein-DNA complex was considered to be similar to that characteristic of the protein itself. This assumption is based on the small size of the DNA used in this experiment.

DNase I-footprinting studies for the identification of the ModE binding sites in the DNA. Although, the ModE titration experiments (Table 7) and mobility shift experiments (Fig. 7) implicated the GTTA tetramers found in the *modA* operator/promoter DNA in the binding of ModE, direct determination of the DNA sequence bound by ModE is required to confirm the involvement of the GTTA tetramers. To this end, DNase I-footprinting experiments were carried out. For one set of DNase I-footprinting experiments, 1 mM sodium molybdate was added to the binding reactions which also included 0.1 pmol of a 446-bp ^{32}P labeled DNA fragment containing the entire *modA* operator/promoter region as well as 1-fold, 10-fold, or 100-fold excess ModE compared to DNA. Analysis of the protection patterns resulting from the DNase I cleavage of the binding reaction mixtures (Fig. 15) reveals that there are three areas of protection. These protected regions are detectable at 10 and 100-fold excess ModE. It is notable that the first protected region

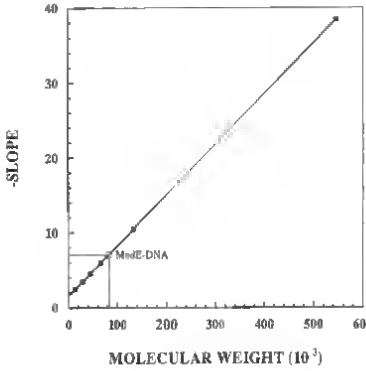

Fig 14 Ferguson plot generated from the R_f values obtained after electrophoresis of the non-denatured protein standards and ModE-43-bp-*modA*-operator/promoter DNA complex in 5% 6% 7% and 8% polyacrylamide TBE gels The dotted lines indicate the extrapolation for the determination of the molecular weight of the ModE-DNA complex form 'A

Fig. 15 DNase I-footprinting experiment in which the binding reactions contained 1 mM sodium molybdate 0.1 pmol ^{32}P-labeled -446 bp-*modA*-operator/promoter DNA and various concentrations of ModE. Lane 1 DNA alone, lane 2 1-fold excess ModE compared to DNA, lane 3 10-fold excess ModE, lane 4 100-fold excess ModE. The boxed sequence represents the nucleotides located in the protected regions. The * indicates bases that are subject to hypersensitivity of DNase I cleavage.

(from top of Fig. 15) (GTTATATTG, -15 to -7) encompasses the fourth and fifth GTTA tetramers located in the -10 region of the *modA*-operator/promoter region (Table 7) which would confirm the importance of GTTA sequences in the binding of ModE. Also there appears to be a 'G' located three bases upstream of this first protected region which is hypersensitive to DNase I-cleavage. However, the second (CCTACAT, -3 to +4) and third (GTTACAT, +8 to +14) protected areas, respectively, do not uphold the prediction of the involvement of the GTTA tetramers located in these regions in the binding of ModE since only part of the sixth and seventh GTTA tetramers (Table 7) which comprise the 8 bp inverted repeat (+4 to +11) are located in the protected area. A second DNase I hypersensitive nucleotide ('A') is located at the lower boundary of the second protected region. The location of these hypersensitive cleavage spots are not unexpected since it has previously been shown that hypersensitive cleavage sites often flank areas of protection (10).

In light of the findings from the DNase I-footprinting experiments, a reevaluation of the sequence involved in binding ModE was undertaken which resulted in the assignment of the sequences TACAT and TATAT as those necessary for the binding of ModE since these sequences are common to all three protected regions. The fact that the TATAT and TACAT sequences found in the three protected regions overlap the fourth and fifth GTTA tetramers (first protected region) and sixth and seventh GTTA tetramers (second and third protected regions) respectively (Fig. 15 and Table 7) supports the interpretation that removal of the above indicated GTTA sequences from certain plasmids used in the ModE titration experiments resulted in the loss of binding by ModE.

A DNase I footprinting experiment was also conducted in which molybdate was withheld from the binding reactions in order to determine whether ModE is capable of binding the DNA, thereby protecting it from cleavage by DNase I, even in the absence of molybdate, and secondly, to determine, if protection does occur, whether the protected regions are identical to those obtained from the reactions supplemented with sodium molybdate. As can be seen from the results of these experiments (Fig. 16), protection of the DNA from DNase I cleavage did occur in the presence of 100-fold and 1000-fold excess ModE, which is ten times more ModE required for the protection in this experiment than was needed when the reactions included sodium molybdate. It was expected that protection of the DNA from DNase I cleavage in the absence of molybdate would be compromised since it had previously been shown in mobility shift experiments (Fig. 9) that the binding of ModE to the 43-bp *modA*-operator/ promoter fragment was severely reduced without the inclusion of sodium molybdate in the binding reactions. The protection pattern that did result from the binding of ModE to DNA in the absence of added molybdate was identical to that obtained from the experiment conducted with sodium molybdate supplementation. However, the G at -38 is hypersensitive to DNase I cleavage only in the presence of molybdate. It is possible that in this experiment, a small fraction of ModE is bound to trace amounts of molybdate present in the solution and that only this ModE-molybdate complex binds to the DNA. Alternatively, molybdate-free ModE can bind to DNA but at a very low efficiency. The differential hypersensitivity of G' at position -38 suggests that molybdate-free-ModE is capable of binding to the DNA.

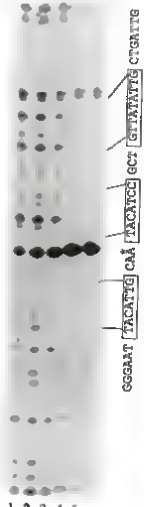

Fig. 16 DNase I footprint of ModE in *modA*-operator/promoter DNA in which sodium molybdate was excluded from the reaction mixtures. The sample in lane 1 contains no ModE; lane 2, 1-fold excess ModE compared to DNA; lane 3, 10-fold excess ModE; lane 4, 100-fold excess ModE; lane 5, 1000-fold excess ModE. The boxed sequences represent nucleotides located in the protected regions. The * indicates nucleotides which are hypersensitive to DNase I cleavage.

The protection of the *modA*-operator/promoter DNA by ModE in three distinct regions is not consistent with the determination of dimeric binding of ModE to DNA in the ModE DNA complex (Fig 14) These results taken together suggest that the association of ModE with the *modA*-operator/promoter DNA is not simply binding of ModE to each site Rather, these data suggest a possible scenario in which a monomeric ModE protein may initially bind loosely to one of the three protected regions, and this initial binding then allows for the rapid recruitment of additional ModE-molybdate to the region for stable binding of ModE protein as a dimer to the other two sites. This monomeric association with the DNA is probably unstable and is rapidly removed by electrophoresis in the DNA-mobility shift experiment. Yet at this point, any further discussion of the course of the binding of ModE to the DNA must await binding experiments carried out and analyzed in solution

In order to evaluate the role of the three ModE-protected regions in vivo, another set of plasmids was constructed for ModE titration experiments similar to the ones previously described (Table 7) Plasmid pAM17 contains protected sites 1 and 2 while plasmid pAM18 has protected sites 1 and 3 with the spacing between the sites preserved (Table 9) Plasmid pAM19 contains only the protected regions 1 and 3 separated by only two bp The *modA - lacZ* expression levels, measured as β-galactosidase activity for strain SE2069 containing the above-mentioned plasmids indicated that only plasmid pAM17 is capable of partially titrating ModE (Table 9) Strain SE2069 harboring plasmid pAM17 produced only 220 β-galactosidase units while only 70 β-galactosidase units were obtained for strain SE2069 lacking any plasmid The level of titration of ModE in

Table 9 Titration of ModE protein in vivo by various regions of the *modA*-operator /promoter DNA in strain SE2069 (*modA*$^{-}$ *lacZ*$^{+}$)

Plasmid	DNA Sequence	β-Galactosidase Activity
No Plasmid		70
pAM4	GGTACC- 186nt —CAAAGTTAGCAATAACTGCTTGGAAAATTCG— GAATTAATCGTATACATGTCGCGTACATAACGTTACATTAAGGGTTAC (1 2 3)	800
pAM17	TCGTATACATGTCGCGTACATAA (1 2)	220
pAM5	CGCGTACATAACGTTACATTAAGGGTTAC (2 3)	80
pAM10	TCGTATACATGTCGCGTTTGGAACGTTACATT (1 3)	60
pAM19	TCGTATATT————————GTTACATT (1 3)	50

Cells were grown in LBG medium with molybdate (1 mM). β galactosidase activities are expressed as nanomoles·minute^{-1}·milligram of cell protein^{-1}. The ModE protein binding sites are indicated by inverse print and the numbers designate the particular binding site (see Text for details). The underlined sequence "CTTGG" replaces the sequence in region 2 preserving the spacing between regions 1 and 3

the cells containing plasmid pAM17 is not the maximal level possible since 600 β-galactosidase units of activity was produced by strain SE2069 with plasmid pAM4 which contains all three protected regions. Thus, it appears that only protected regions 1 and 2 are absolutely required for the binding of ModE but that in addition, region 3 is necessary for maximal binding.

DNA mobility shift experiments were also conducted using the *modA*-operator/promoter DNA contained in plasmids pAM17, pAM18, and pAM19. As would be expected, based on the in vivo titration experiments, only the *modA*-operator/promoter DNA present in plasmid pAM17 was capable of forming the A complex in the presence of ModE protein as presented in Fig. 17 (Panel I). Moreover, as is also suggested by the partial titration seen in the ModE titration experiments, the half-maximal binding of ModE to the 36-bp *modA*-operator/promoter fragment occurs at a ModE concentration of 1.8 μM (Fig. 18) which is considerably higher than the 0.33 μM apparent K_D value obtained for the binding of ModE to DNA containing all three sites (Fig. 10). The results of the in vivo ModE-titration experiments coupled with the in vitro DNA-mobility shift experiments indicate that the binding of ModE only requires sites 1 and 2, but site 3 is necessary for efficient binding at sites 1 and 2. These data lend credence to the premise that site 3 may serve as a location to which ModE initially binds, thereby promoting the stable binding of ModE to sites 1 and 2.

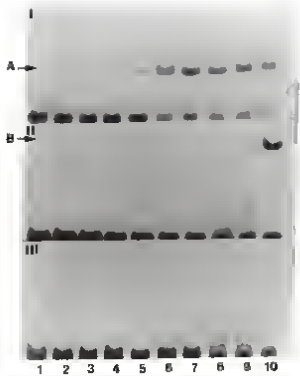

Fig. 17 DNA mobility shift experiments using the *modA*-operator/promoter DNA contained in plasmids pAM17, pAM18, and pAM19 and ModE. For the shift with DNA present in plasmid pAM17 (panel I), a ^{32}P labeled 36-bp *modA* DNA fragment was included in the binding reactions along with 1 mM sodium molybdate and the appropriate amount of ModE (lane 1, without ModE; lane 2, 1-fold excess ModE compared to DNA; lane 3, 5-fold excess ModE; lane 4, 10-fold excess ModE; lane 5, 25-fold excess ModE; lane 6, 50-fold excess ModE; lane 7, 100-fold excess ModE; lane 8, 250-fold excess ModE; lane 9, 500 fold excess ModE; lane 10, 1000 fold excess ModE). The mobility shifts featuring the 45-bp *modA* DNA present in plasmid pAM18 (panel II) and 31-bp *modA* DNA present in plasmid pAM19 (panel III) also contained 1 mM sodium molybdate and the amount of ModE indicated for the lanes in panel I.

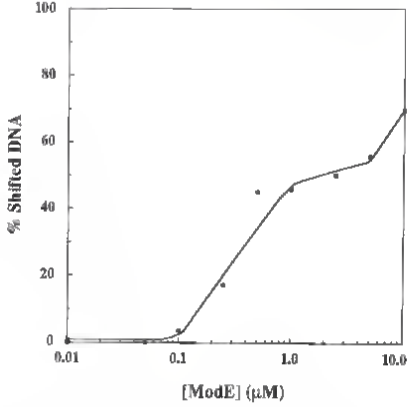

Fig. 18 Plot of % shifted DNA versus concentration of ModE required for the shift featuring the 36-bp *modA* DNA present in plasmid pAM17. The data were from the experiment presented in Fig. 17 Panel I

Molybdate-independent ModE Proteins

Mutation of DNA which codes for protein of interest is often employed as an approach to study structure/function characteristics of the protein. In this study hydroxylamine, which causes G-to-A transitions, was used to mutate the DNA coding for ModE (plasmid pAG1) in order to identify critical amino acids responsible for the association of molybdate with ModE and/or the binding of ModE to its target DNA. Following the hydroxylamine treatment, two different mutant phenotypes were observed: partial loss of repression of the *modABCD* operon in the presence of molybdate and complete repression of the *modABCD* operon in the absence of molybdate (superrepressor). The phenotypes observed were confirmed by assaying strain SE1811 harboring plasmids with *modE* mutations for β-galactosidase activity, and the results of these experiments are presented in Fig. 19.

The partially derepressed (A76V) ModE mutant has a substitution of valine for alanine at amino acid position 76. Three "superrepressor" mutants which were capable of repressing the *modABCD* operon in the absence of molybdate were also isolated, and two of these mutants harbor the following mutations: threonine at amino acid position 125 replaced with isoleucine (T125I) and glycine at position 133 changed to aspartic acid (G133D). For the third "superrepressor" mutant (Q216*) the C-terminal 47 amino acids starting from the amino acid at position 216 were deleted.

Plasmid	Mutation	β-galactosidase Activity -Mo	+Mo
pAG1		600	100
pAG1(A76V)	A76V	1 300	600
pAG1(T125I)	T125I	125	110
pAG1(G133D)	G133D	40	40
pAG1(Q216*)	Q216*	<10	<10

Fig 19 Analysis of ModE mutants. β-galactosidase activity produced by strain SE1811 carrying the indicated mutation was determined and expressed as nanomoles min^{-1} milligram of cell protein. Cells were grown in LBG with (+) or without (–) sodium molybdate (Mo) at a final concentration of 1 mM. * represents translation termination.

The mutation in the partially derepressed mutant (A76V) lies immediately after a stretch of three glycines which are identical in three of the four ModE homologs presented in Fig. 3 the fourth (RcMopA) having two of the three glycines. Two of the four homologs (EcModE and RcMopA) have alanine at this position while the other two (AvModE and HiModE) have threonine at this position. Furthermore this mutation is 23 amino acids away (towards the C-terminal end) from the putative helix-turn-helix structure, so it is possible that this mutation may have disrupted the helix-turn-helix structure resulting in a partial loss of DNA binding by the mutant protein

Two of the "superrepressor mutants" T125I and G133D have mutations situated near a five amino-acid long sequence SARNQ (amino acids 126 to 130), which is conserved among the five homologs, with the exception of *R. capsulatus* MopA which has an asparagine in place of alanine and a threonine in place of glutamine. In fact, the T125I mutation directly precedes the SARNQ sequence and is itself conserved among three of the homologs (AvModE, EcModE and RcMopA) with serine substituting for threonine in *H. influenzae* ModE. The G133D mutation lies two amino acids further on the C terminal side of the SARNQ sequence and the glycine is also conserved in four of the homologs (AvModE EcModE HiModE and CpMopII) The glutamine which is replaced by a stop codon in the third superrepressor mutant (Q216*) is unique to *E. coli* ModE at this position, however there are regions of conserved amino acids present in the missing C-terminal end of ModE whose loss undoubtedly contributes to the phenotype of this mutant

It is also interesting to note that the amino acid sequence deleted in this mutant (Q261*) contains the only three cysteines present in the wild type *E. coli* ModE protein, and it is possible that two of these three cysteines may be involved in forming a disulfide bond. Moreover, it is tempting to speculate that ModE adopts an open conformation when molybdate is not bound to the protein, and that upon binding of molybdate a change in the structural conformation ensues which involves formation of a disulfide bond. This formation of the disulfide bond could stabilize a more compact structure which promotes the efficient binding of ModE to the appropriate DNA sequences. Furthermore, it is apparent that the deletion of the region of the protein which contains these cysteine residues in the (Q216*) mutant ModE protein resulted in the adoption of the "activated" or molybdate-bound conformation even in the absence of bound molybdate.

At the current level of information available concerning the structure of the ModE protein, a more defensible assertion at this point in time is that the SARNQ sequence may be integral to the binding of molybdate by ModE given that two of the three isolated "superrepressor" mutations abut this SARNQ region and that all of the ModE homologs as well as other proteins that are either known to have or are suspected to have interactions with molybdate contain variations of this sequence (Table 10). It should also be mentioned that it would be expected that binding of the negatively charged molybdate moiety by ModE would most likely require positively charged amino acids such as those present in the SARNQ region. Furthermore, if the nature of the G133D "superrepressor" mutation is considered, it is possible that the substitution of aspartic acid in place of

Table 10 List of proteins which are known to interact with molybdate and DNA and contain a possible molybdate-binding motif

Protein	Molybdate-binding motif sequence	Reference
ModB from E coli	SARNQ	(24,47,66)
ModE from H influenzae	SARNQ	(20)
ModE from A vinelandii	SARMQ	(43)
MopII from C pasteurianum	SARNQ	(32)
MopA from R capsulatus	SnRNt	(76)
PhlA from E coli	SGRNN	(47 64)
NarX from E coli	SGRNe	(36)
NarQ from E coli	SlReQ	(12)
Estrogen receptor from Rat	LdRNQ pAtNQ	(44)
Estrogen receptor from Human	LARgQ	(33)

Lower case letters represent non-identical or non-similar amino acids NarX and NarQ are membrane-bound components of the *nar* regulatory system but do not bind DNA themselves

glycine mimics the binding of molybdate due to the presence of the negatively-charged carboxyl group in the amino acid side chain.

A listing of regulatory proteins which interact with molybdate and corresponding SARNQ-like sequences found in these proteins are presented in Table 10. As has already been indicated, four of the five ModE homologs contain the SARNQ sequence, while *R. capsulatus* ModE homolog, MopA, has an asparagine in place of alanine and threonine in place of glutamine. FhlA which serves as a molybdate-responsive activator of the *hyc* operon which codes for hydrogenase 3 (47, 63, 64, 68) has a sequence SGRNN (amino acid positions 426 to 430) which has three of five conserved amino acids while the remaining two are similar. NarX protein, which functions to activate the regulator protein, NarL, in response to the presence of nitrate, contains the sequence SGRNE (amino acid positions 204 to 208, 38). Interestingly, specific mutations in the SGRNE region result in the molybdate-independent activation of NarL (38). A second nitrate sensor protein, NarQ, has the sequence SLRMQ (amino acids positions 48 to 52, 12) which bears similarity to the general motif. Furthermore, the rat estrogen receptor-hsp90 complex which is stabilized by molybdate (44) contains two sequences which share similarity with SARNQ, one defined as PATNQ (amino acid positions 227 to 231) and a second LDRNQ which is located at amino acid positions 415 to 419 (44). The human estrogen receptor also contains a sequence similar to SARNQ, LARGQ located at amino acid positions 190 to 194 (39). However, this SARNQ sequence or any similar sequence is not found in any of the periplasmic molybdate binding proteins of the transport system.

The fact that certain mutations affecting amino acids located near the SARNQ sequence result in the molybdate-independent phenotype for ModE suggests that these amino acids are important for the proper functioning of the ModE protein. Secondly, the fact that all of the ModE homologs and a number of proteins which are thought to interact with molybdate contain sequences which are either completely conserved or bear striking similarity to the SARNQ amino acid sequence further implies that the amino acids in this region may be involved in activation of the ModE aporepressor by means of binding molybdate.

Regulation of *modE*

The regulation of *ModE* was investigated by introducing φ(*modE - lacZ*) borne on λRM26 into various backgrounds and assaying for β-galactosidase activity. The results presented in Table 11 indicate that there is little difference in the levels of β-galactosidase obtained from a wild type strain, SE1938, a *modB* mutant strain, SE1952, a *modE* mutant strain, SE1942, or a *modF* mutant strain, SE1940. These results show that *modE* is transcribed constitutively at low levels, which is consistent with ModE's proposed role as a repressor.

A plasmid-based φ(*modE - lacZ*), plasmid pRM14, was used to determine whether mutations in global regulators such as *arcA* and *narL* gene products would elicit changes in the expression of *modE* (Table 12). Similarly, mutations in genes required for molybdate incorporation into molybdopterin, *moe*, *moh*, *moe*, and *meg* were analyzed for their effect on *modE* transcription levels. No significant change in expression was

Table 11 Regulation of *modE* in different *mod* strains

Strain	Relevant Genotype	β galactosidase activity[a] −Mo	+Mo
SE1938	ϕ(*modE - lacZ*)	324	297
SE1952	ϕ(*modE - lacZ*) *modB*::Tn5	316	249
SE1942	ϕ(*modE - lacZ*) Δ(*modE* Km)	351	324
SE1940	ϕ(*modE - lacZ*) *modF*::km	378	351

[a]Cells were grown in LBG medium with or without sodium molybdate at a final concentration of 1 mM. Enzyme activities are expressed as nanomoles min^{-1} milligram of cell protein

Table 12 Regulation of *medE* in various backgrounds

Strain	Relevant Genotype	β-galactosidase activity[a]	
		-Mo	+Mo
RK4353(pRM14)	ϕ(*modE - lacZ*)	11 860	11 400
ECL618(pRM14)	ϕ(*modE - lacZ*) *arcA*	12 350	10 000
RK5278(pRM14)	ϕ(*modE lacZ*) *narL*	15,500	15 500
VJS1782(pRM14)	ϕ(*modE - lacZ*) *moa*	10,950	10,450
VJS1780(pRM14)	ϕ(*modE - lacZ*) *mob*	10,300	13,400
VJS1779(pRM14)	ϕ(*modE - lacZ*) *moe*	10,350	12,300
VJS1784(pRM14)	ϕ(*modE - lacZ*) *mog*	12,000	11,850

[a]Cells were grown in LBG medium with or without sodium molybdate at a final concentration of 1 mM. Enzyme activities are expressed in nanomoles min^{-1} milligram of cell protein^{-1}

observed in any of these backgrounds. The higher β-galactosidase activity in these plasmid containing *modE - lacZ* strains is a consequence of multiple copies of the *modE - lacZ* fusion.

Regulation of Other Genes by ModE

ModE has already been shown to exert a negative effect on the transcription of the *modABCD* operon when sufficiently high amounts of molybdate are present in the cell. Investigation of the effect of ModE on the transcription of other genes has led to findings that ModE also regulates transcription of *modF* as well as *narG*. The β-galactosidase activities produced by a *modF - lacZ* operon fusion (λRM37) in the λ *att* site, which demonstrate the regulation of *modF* by ModE, are presented in Table 13. Since *modE* and *modF* appear to comprise a two-gene operon transcribing from a single promoter through both genes, the study of the regulation of *modF* by *ModE* required the uncoupling of the genes so that expression of *modF* can be established without interference by ModE produced from the same operon. This uncoupling of the genes was accomplished by removal of a segment of the *modE* gene from plasmid DNA containing the ϕ(*modF'-lacZ*) before recombining (in-frame deletion) with phage λRZ5 to produce λRM37 thereby preventing production of functional ModE protein, but leaving the entire *modF* gene in frame with respect to the *modEF* promoter.

Production of β-galactosidase activity by a strain carrying *modF - lacZ* and a functional chromosomally encoded ModE protein (Strain SE2105) is very low (70 β galactosidase activity units) as compared to 1,050 β galactosidase activity units obtained for the *modE* mutant strain (strain SE2106). Introduction of a plasmid containing wild

Table 13 Regulation of *modF* by ModE

Strain	Relevant Genotype	β-galactosidase activity[a]	
		-Mo	+Mo
SE2105	ϕ(*modF lacZ*)	70	70
SE2106	ϕ(*modF lacZ*) Δ*modE*-km	1 050	1 180
SE2106 (pRM20)	ϕ(*modF lacZ*) Δ*modE*-km, *modE*⁺	<10	<10
SE2107	ϕ(*modF* '*lacZ*) Δ*modF* km	180	170
SE2112	ϕ(*modF lacZ*) Δ*modEF*-km	2,730	2,160
SE2114	ϕ(*modF lacZ*) *modB*::Tn10	1,190	30
SE2116	ϕ(*modF lacZ*) Δ*modE*-km, *modB*::Tn10	1 080	990
SE2118	ϕ(*modF lacZ*) Δ*modF* km, *modB*::Tn10	1 030	31

[a]Cells were grown in LBG medium with or without sodium molybdate at a final concentration of 1 mM. Enzyme activities are expressed as nanomoles min^{-1} milligram of cell protein.

type *modE* (plasmid pRM20) into the *modE* mutant strain SE2106 restores the repression of *modF - lacZ* expression as evidenced by the very low β-galactosidase activities (< 10) obtained for strain SE2106(pRM20). Expression of *modF - lacZ* was approximately 2 times greater in a *modF* mutant strain (strain SE2107) than in the wild type strain. This higher level of expression was also observed for the strain deficient in both ModE and ModF (strain SE2112) compared to the strain deficient only in ModE, suggesting that *modF* expression may be subject to autoregulation as well as repression by ModE.

As would be expected, given that in vivo as well as in vitro experiments have indicated that the DNA binding capacity of ModE depends on the presence of molybdate, repression of *modF - lacZ* expression was alleviated in molybdate transport mutant strains (strains containing *modB::Tn10*). Supplementation of the growth medium with 1 mM sodium molybdate, restored repression of *modF – lacZ* expression in all the molybdate transport mutant strains except for the *modE modB::*Tn10 double mutant strain (strain SE2116).

Based on the data presented in Table 13, it appears that ModE represses the transcription of *modF* when there is a sufficient intracellular concentration of molybdate. Moreover, the regulation of *modF* by ModE is similar to the regulation of the *modABCD* operon by ModE (Tables 3 and 4). This similarity in the regulation of both *modF* and *modABCD* by ModE is not surprising, since it is likely that the functions of all of the effected genes are in some way directed towards the successful transport of molybdate and incorporation of activated molybdenum into the appropriate apo-molybdoenzymes, and as such would most likely be coordinately regulated.

The regulation of *narG* by ModE on the other hand was somewhat unexpected since it has already been shown that molybdate exerts influence over the transcription of *narG* presumably through the NarX and NarQ proteins and an additional level of molybdate dependent control through ModE seems superfluous (12, 38). However as can be seen by the levels of *narG - lacZ* expression presented in Table 14 ModE clearly has an effect on transcription through *narG*. More specifically the absence of ModE results in reduction of transcription through *narG - lacZ*, as evidenced by the drop in β-galactosidase activity from 28 280 units in the wild type strain to 11 010 units in the *ModE* strain. As would be expected, complementation of the *modE* mutation by introduction of a *modE* containing plasmid pAG1 into the *modE* mutant strain resulted in an increase in the β-galactosidase activity to a value comparable to wild type levels. The nature of the regulation of *narG* exerted by ModE is opposite of that seen for either the *modABCD* operon or for *modF* as it appears this ModE is functioning as an enhancer of activation of *narGHJI* operon expression. This shows the complexity of the regulation of anaerobic respiratory pathways in this case the regulation of *narG* expression has already been shown to be positively activated by NarL as well as FNR. Apparently the additional level of regulation afforded by ModE helps optimize the expression of *narG* operon in response to molybdate availability.

The possibility of ModE serving as an activator for certain genes is currently being explored for the *hyc* and *maeAB* operons (27, 68). Yet at this juncture it is premature to state that the ModE functions as both a repressor of the *modABCD* operon and *modF* as well as an activator of other genes until studies providing direct proof of ModE's

Table 14 Regulation [illegible] expression by ModE

Strain	Relevant Genotype	β-galactosidase activity
MC4100(λPC50)	φ(*narG - lacZ*)	28,280
SE4110(λPC50)	φ(*narG - lacZ*) Δ*modE*-km	11,010
SE4110(λPC50) (pAG1)	φ(*narG - lacZ*) Δ*modE*-km, *modE*$^+$	33,460

[a]Cells were grown in LBG medium supplemented with 40 mM potassium nitrate and 1 mM sodium molybdate. Enzyme activities are expressed as nanomoles min^{-1} milligram of cell protein^{-1}

interaction with these other genes (binding studies, footprinting experiments, etc) have been completed.

CONCLUSION

Proper function of the complement of molybdoenzymes present in *E. coli* requires the synthesis of the molybdopterin guanine dinucleotide form of the molybdenum cofactor. The successful production of the molybdenum cofactor is predicated on the presence of an intracellular pool of molybdate. In the typical *E. coli* habitat, only trace amounts of molybdate are likely to be found. This low level of molybdate in *E. coli*'s environment necessitates the existence of a high-affinity molybdate-specific transport system in *E. coli*. Such a transport system has been identified and is coded by the *modABC* genes.

However, a number of considerations such as the fact that *E. coli* possesses only a few enzymes requiring molybdenum and that the majority of those enzymes are membrane-associated and are, therefore, sparingly produced in the cell, and the fact that at high intracellular concentrations, molybdate is toxic to the cell would suggest that the molybdate transport system be regulated in response to molybdate availability. Indeed, expression of the molybdate-specific transporter genes was found to be negatively regulated under conditions of intracellular molybdate saturation. Isolation of an *E. coli* strain (strain SE1811) which did not exhibit molybdate-induced repression of the *modABCD* operon led to the identification of a protein, designated ModE, which is responsible for the molybdate-dependent repression of transcription of the transport genes.

Subsequent studies confirmed the initial assignment of repressor function to ModE by demonstrating that ModE in the presence of 1 mM molybdate, substantially reduced the production of plasmid-encoded ModA and ModB proteins in a coupled transcription-translation system. Further supporting evidence was provided by the successful binding of ModE to DNA derived from the operator/promoter region of the *modA* sequence in DNA mobility shift experiments. Also, the DNA-mobility shift experiments showed that the binding of ModE to its target DNA required molybdate since an apparent K_D of 0.22 μM was calculated for the binding of ModE to DNA in the presence of 10 μM molybdate, whereas binding in the absence of molybdate did not occur to an extent which would allow calculation of a K_D value. It was also shown that half-maximal binding of ModE to *modA* operator/promoter DNA resulted when 6 μM molybdate was present in the binding reaction. The difference in the dissociation constants for the binding of ModE to the DNA (0.22 μM) and the apparent dissociation constant of molybdate to ModE (6 μM) suggests that the binding of molybdate to the ModE protein serves as the limiting step in the interaction of ModE with the *modA* operator/promoter DNA.

DNase I-footprinting experiments revealed that ModE binds to the *modA* operator/promoter DNA at three locations, and that the sequences "TATAT" and "TACAT", present in these DNase I-cleavage-protected regions, constitute the ModE recognition sequences. The fact that the ModE-DNA complex formed in the DNA-mobility shift experiments consists of only a ModE dimer bound to the 43 bp *modA* DNA implies that ModE is forming only a loose association with one of the three DNase I cleavage-protected regions, and that this loose association promotes the subsequent stable

dimeric binding of ModE to the other two binding sites. This possible binding scenario was supported by ModE titration experiments and the corollary DNA-mobility shift experiments (Table 9 and Fig. 17) which indicate that the first and second binding sites are minimally required for ModE binding, but that efficient binding requires the presence of all three sites; however further studies which can discriminate the order of binding need to be conducted to confirm this particular scenario for the binding of ModE to the DNA.

Isolation of the T125I and G133D mutant ModE proteins which are capable of repressing the *modABCD* operon in the absence of molybdate, was crucial to the identification of the TSARNQXXG amino acid sequence (or variations thereof) that is present in the *H. influenzae A. vinelandii R. capsulatus* and *C. pasteurianum* ModE homologs. Similar amino acid sequences were also found in other proteins that are known to interact with molybdate suggesting that this stretch of amino acids is most likely involved in the binding of molybdate by the ModE protein as well as in the other proteins. Although, the mutation analysis has provided some information in terms of structure/function, confirmation of the interpretations of these results requires X-ray crystallographic or NMR based structural studies.

Lastly *modE* expression was shown to occur constitutively at low levels which is characteristic of repressor proteins that require modification for function. However, ModE, itself, was also shown to regulate additional genes other than the *modABCD* operon when it was demonstrated that ModE negatively regulates *modF* in a molybdate-dependent fashion but enhances the expression of *narG*. Preliminary studies also indicate that ModE enhances the transcription of *moeA* and *hycA* genes as well (27, 68). Thus it

appears that ModE functions primarily to repress the expression of the *modABCD* genes in a molybdate-dependent manner but additionally may enhance the transcription of select genes which are involved in molybdate metabolism in order to optimize *E. coli*'s biosynthetic and energy producing systems under varying conditions

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy

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